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Multiple methods were used to evaluate the role of bacterial endotoxin(LPS) in the observed toxicities of solutions of hemoglobin(Hb). A series of physical measurements were performed to determine the effects of Hb on the LPS macromolecule. These included ultrafiltration, density centrifugation, precipitation, and gel filtration, as well as investigations specifically designed to evaluate binding of LPS by Hb and to determine whether complex formation occurs. The data support the conclusion that the interaction between LPS and Hb results in the formation of a complex and that Hb is an LPS binding protein. The formation of LPS-Hb complexes is associated with disaggregation of the LPS macromolecule and importantly, with marked enhancement of the biological activity of LPS. Enhancement of the biological activity of LPS has been shown in two independent biological systems, i.e., activation of the coagulation cascade of Limulus amebocyte lysate and stimulation of the production of tissue factor by human endothelial cells. Similar results have been shown for a wide variety of clinically relevant LPSs and for both purified, native HbA_0 and $\alpha\alpha$-crosslinked Hb. Conversely, LPS increased the rate of oxidation of Hb, with formation of MetHb and/or hemichromes. Importantly, administration of Hb increased the mortality produced in mice by LPS.

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Cell-free hemoglobin(Hb); Bacterial endotoxin(LPS); Bacterial lipopolysaccharide(LPS); LPS-Hb complexes; LPS disaggregation; LPS, enhanced biological activity; $\alpha\alpha$-crosslinked Hb($\alpha\alpha$Hb)

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Jack Levin 4/1/99

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FINAL SCIENTIFIC REPORT

Introduction

The development of solutions of hemoglobin (Hb) for use in both military and civilian settings remains an important goal. However, an important limitation for the utilization of Hb is potential in vivo toxicity. Toxicities of hemoglobin (Hb) solutions, described in numerous animal resuscitation models, have prominently included fever, hypertension, thrombocytopenia, activation of the complement and coagulation cascades, disseminated intravascular coagulation with parenchymal organ damage, vasculitis with resultant hemorrhagic lesions, reduced tolerance to sepsis, susceptibility to bacterial infections, reticuloendothelial cell blockade and lethal toxicity (1-13). Many of these effects (most of which were reported prior to 1991) may have been due to impure or bacteriologically contaminated preparations of Hb. Recent clinical trials of cross-linked Hb have not demonstrated most of these toxicities, but have been associated with production of hypertension and gastrointestinal dysmotility.

It was recently demonstrated that injection of non-lethal doses of gram-negative bacteria into mice produced 50% and 100% mortality when the animals had been pre-infused with either native or cross-linked preparations of cell-free Hb, respectively (14). Mortality was lessened when sepsis was not produced until 3 hours after administration of either Hb. However, these results

were not confirmed in apparently similar models which either were performed with mice (15) or rats (16). In vitro, Hb has been shown to stimulate tissue factor production by mononuclear cells (17), cause endothelial cell injury (18) and to activate complement (17). These in vivo and in vitro effects are characteristic of bacterial endotoxins (lipopolysaccharide, LPS). Investigations of the possibility that LPS may contribute to the observed side effects of Hb infusions have been a major focus of our laboratory during the past several years, and a significant role for LPS in Hb toxicity has been suggested by our studies.

One of the most critical aspects of LPS toxicity is the high in vivo potency of LPS, even at very low concentrations (pg/ml). LPS is a potentially ubiquitous contaminant during the preparation of Hb-based resuscitation fluids, and even low levels of LPS contamination become a major clinical concern when large volumes of resuscitation solutions are required for infusion. In addition, physiologically significant levels of LPS are present in the circulating blood in a variety of clinical conditions, including sepsis, hepatic injury, hypotension, and damage to the gastrointestinal tract. Because many clinical circumstances for which Hb-based resuscitation fluids would be administered are likely to be associated with shock and hypoxia (pathological states that lead to deterioration of mucosal barriers and hepatic function), significant concentrations of endotoxin would be expected to be potentially present in the circulation of many patients. Since there is increasing evidence that cell-free Hb and LPS synergistically produce toxicities, the infusion of Hb-based resuscitation fluids

may potentiate the toxicity of pre-existing endotoxemia (or of endotoxemia that subsequently occurs when Hb remains present in the plasma), thus compounding the problem of the high intrinsic biological potency of LPS. The co-infusion of LPS and Hb into rabbits activated blood coagulation and produced a marked increase in mortality (50-100%) compared to the toxicity of LPS or cell-free Hb alone (only 0-10%) (19). A similar increase in mortality was produced in rabbits by the intravenous administration of LPS and αα-crosslinked Hb (20). We have shown (following sections) that LPS clearance in vivo is retarded in the presence of hemoglobinemia. LPS biological effects in vitro, such as activation of coagulation mechanisms (both the direct activation of coagulation cascades and the production of monocyte and endothelial cell-derived procoagulant activity), can be enhanced up to one-hundred fold by cell-free Hb. Furthermore, rates of Hb oxidation to methemoglobin and hemichromes are dramatically increased in the presence of LPS. Thus, the binding of cell-free Hb to LPS produces complexes that result both in enhancement of the biological activities of LPS and degradation of Hb.

Investigations in our laboratory with Hb solutions, including both native human HbA_0 and cross-linked Hb (human Hb, αα cross-linked using bis(3,5-dibromosalicyl) fumarate [DBBF]; and bovine Hb, fumaryl ββ cross-linked), have led to an understanding of the complex contributions of LPS to the observed toxicities of Hb solutions. Initial experiments suggested the possibility that Hb was a previously unrecognized LPS binding protein. Subsequently, detailed experiments documented the formation of Hb-LPS

complexes, characterized the complexes, and identified consequences of the LPS-Hb interaction that might contribute to toxicity.

Body(Experimental Methods, Results, and Discussion)

Demonstration that Hb is an LPS binding protein.

An extensive series of experiments has been utilized to document that mixtures of LPS and Hb produce stable complexes (21). In all experiments, equivalent results were obtained using either purified native, unmodified human HbA_0 or cross-linked Hb prepared as a potential red cell substitute. Direct evidence of saturable binding of LPS to immobilized Hb was obtained (Fig. 1). The calculated K_d (4.7×10^{-4} g/liter [3.1×10^{-8} M, assuming a monomer molecular mass of 1.5×10^4 for *E. coli* LPS] based on the microtiter plate binding assay and 6.3×10^{-4} g/liter based on a sucrose centrifugation assay) indicated that the interaction between Hb and LPS is of moderate affinity. Complex formation also was demonstrated by affinity-labeling of Hb with a photoactivatable form of LPS (Fig. 2). Using density gradient centrifugation, co-migration of LPS with Hb was shown, and it was demonstrated that the sedimentation velocity of LPS was decreased in the presence of Hb preparations (Fig. 3). This indicated that there had been disaggregation of LPS and formation of lower density Hb-LPS complexes. Additional evidence of LPS dissociation was obtained by non-denaturing polyacrylamide gel

electrophoresis which demonstrated that LPS, when complexed with Hb, entered the gel and co-migrated with Hb, whereas LPS alone remained within the stacking gel (Fig. 4). Ultrafiltration experiments demonstrated that LPS, which alone in aqueous solutions has a very high molecular weight (typically $\geq 10^6$ daltons), co-filtered with Hb through 300 kDa and 100 kDa membranes (Table 1). Whereas only 10-16% of LPS alone was filterable through the 300 kDa membrane and LPS alone was not filterable at all through the 100 kDa membrane, in the presence of Hb, 87-97% of LPS was filtered through the 300 kDa membrane and 64-72% through the 100 kDa membrane. Thus, these data provide further evidence that Hb greatly decreased the aggregate molecular weight of LPS.

Independent evidence that Hb is an LPS binding protein has been provided by recent investigations that demonstrated binding of porcine Hb to the LPS of *Actinobacillus pleuropneumoniae* as well as binding to the surface of intact bacteria of this species (22). In addition, the LPS was shown to bind to both the α and ß chains of porcine Hb (22), confirming our observations that LPS binds to both the α and ß chains of human Hb (21).

As described above, Hb alters several characteristics of LPS. Conversely, LPS can produce Hb denaturation, with production of methemoglobin and hemichromes (Fig. 5)(23). Degradation of Hb by LPS is time (Fig. 6) and LPS-concentration dependent. There also are structural changes indicative of Hb oxidation as demonstrated by circular dichroic analysis between 210-600 nm.

However, there is no demonstrable change in the overall tertiary structure of the globin molecule (23).

P50 measurements. The oxygen affinity of Hb was measured in the absence and presence of LPS in order to evaluate the possible influence of LPS binding on Hb function (Table 2)(23). These measurements were made after a 2 hr incubation period, a time sufficient to result in Hb-LPS complex formation (24), but prior to the formation of substantial quantities of oxidized Hb species unable to bind oxygen. 1 mg/ml Hb (16 μM) and 1 mg/ml of each LPS were utilized because the two components of Hb-LPS complexes are of approximately equal concentration by weight and little unbound Hb is calculated to be present. P_{50} values for ααHb (26.6 mm Hg) and HbA_0 (9.6 mm Hg) were slightly decreased by both smooth and rough LPSs (Table 2). Non-cross-linked cell-free HbA_0, which exhibited high oxygen affinity (P_{50} = 9.6 mm Hg) similar to that measured with lysed whole blood (P_{50} = 10.0 mm Hg; data not shown), best demonstrated the small trend toward higher oxygen affinity when in the presence of LPS (P_{50} = 7.3 mm Hg in the presence of OH37 LPS). Thus, there is little change in oxygen affinity of Hb when complexed to LPS.

Effect of Hb on LPS clearance in vivo.

LPS clearance in rabbits was shown to be delayed in the presence of Hb (free Hb levels were 2 g/dL, which produced a 15% increase in total circulating Hb) compared to LPS clearance in animals given equivalent doses of human serum albumin (HSA) or NaCl

(Fig. 7)(25). The intravascular retention of injected ^{125}I-LPS during the 30 min period analyzed was significantly longer in the LPS + Hb group than in the LPS + NaCl or LPS + HSA groups, especially during the initial 10 min. The intravascular half-life ($T_{1/2}$) of LPS in the LPS + NaCl control, LPS + HSA control, and LPS + Hb groups was 2.8, 4.0, and 4.9 min; the area under the curve was 1,369 ± 483, 1,594 ± 360, and 1,731 ± 481 (ng/ml x min, mean ± SD); and the total body clearance was 24.7 ± 9.2, 20.1 ± 5.4, and 18.9 ± 6.0 (ml/min, mean ± SD), respectively. The proportion of LPS associated with blood cells was very small at the initial 1 min time period, and decreased even further during the 30 min period analyzed. Over 96% of injected LPS was associated with the cell-free plasma, with 51-54% of LPS in the apoprotein fraction at the initial time point, and 35-37% in the high density lipoprotein (HDL) fraction. The proportion of LPS increased significantly in the HDL fraction and decreased significantly in apoproteins during the 30 min period analyzed. However, there were no differences between the three groups (25, 26). The liver was the main distribution site (74%) of injected LPS, among the six organs evaluated(liver, kidney, lung, spleen, adrenal, and heart). In the Hb group, the accumulation of ^{125}I-LPS in the spleen was significantly lower than in the HSA group. The synergism of the in vivo toxicity reported for LPS and Hb may be due, in part, to the decreased rate of intravascular clearance of endotoxin.

Demonstration that Hb enhances the biological activity of LPS.

Limulus amebocyte lysate activation. The effect of Hb on the biological activity of LPS was initially investigated using Limulus

amebocyte lysate (LAL), the most sensitive in vitro assay for LPS. LAL, a preparation from the blood cells of the horseshoe crab *Limulus polyphemus*, contains an LPS-activated coagulation cascade that is sensitive to pg/ml concentrations of LPS. Each of a variety of LPS preparations obtained from *Proteus mirabilis* and spiked into solutions of LPS-free Hb demonstrated greatly increased activation of LAL, using a chromogenic endpoint, in comparison to identical concentrations of LPS assayed in saline (Fig. 8)(24). Similar results were obtained with endotoxin obtained from *Salmonella minnesota* 595 and with purified Lipid A (24). Furthermore, all three Hb preparations tested ($\alpha\alpha$Hb, HbA_0, and $\alpha\alpha$HbCO) produced enhanced activation of LAL over a wide range of LPS concentrations (Fig. 9). The enhancement by each Hb of LAL activation also was demonstrated with the gelation LAL test, and was shown to be dependent on protein concentration (Fig. 10). LPS biological activity was enhanced >1000-fold at the concentrations of Hb that would be achieved in vivo for purposes of resuscitation. Pertinently, similar Hb concentrations have been occasionally detected in plasma following hemolysis associated with endotoxemia(27). These results are of great interest because LAL activation is an excellent model for the intravascular coagulation which is commonly seen in humans during endotoxemia and which has been described repeatedly during infusion of hemoglobin solutions in animals.

In order to further establish the generalized nature of the Hb enhancement effect, we studied the effect of $\alpha\alpha$Hb on biological activities of several other LPSs, including LPSs from different

bacterial species. Prominent and identical extents of enhancement by both αα Hb and αα HbCO in the LAL assay were shown with three defined salts of *E. coli* 026:B6 (smooth LPS), i.e., the calcium, sodium and triethylamine forms, suggesting that the specific cations bound to LPS did not influence the Hb enhancement process. Enhancement of LPS biological activity also was demonstrated with a smooth *Salmonella* LPS (*S. abortus equi*) and a rough *E. coli* LPS (Re F515), but was not observed with non-toxic *Rhodobacter spheroides*, *Rhodobacter capsulatus* and *Rhodopseudomonas viridis* LPSs.

Many of the LPS preparations studied had poor aqueous solubility and were visually turbid (especially *S. minnesota* 595 LPS, lipid A and monophosphoryl lipid A, and *P. mirabilis* R110). Hb enhancement of LPS biological activity was a prominent feature of some of these LPSs and their partial structures, suggesting that a possible mechanism for the Hb enhancement effect was via increased LPS solubility. Therefore, we compared turbidity and the LAL biological activity of these LPSs in the absence and presence of Hb (24). With increasing concentrations of αα Hb, *P. mirabilis* R110 and *S. minnesota* 595 LPS each demonstrated a concomitant progressive decrease in turbidity and increase in LAL biological activity (Fig. 11).

To further demonstrate the effect of Hb on the physical state of LPS, electron microscopic studies were performed. In the absence of Hb, *S. minnesota* (Re) 595 LPS was highly aggregated, and consisted of variable ribbon-like, mesh-like, and/or membrane-like structures, with the largest dimensions greater than 1 mm (Fig. 12). However, in the presence of HbA_0, marked disaggregation of all of the highly

aggregated LPS structures was demonstrated, with production of discoidal 5-20 nm particles (Fig. 13)(28). Similar results were shown with LPS from E. coli (Re) F515. Recent investigations with *Actinobacillus pleuropneumoniae* LPS and porcine Hb have confirmed that Hb causes disaggregation (and decreased density) of LPS(29).

Tissue factor production. To further investigate the ability of Hb to modify LPS-activated coagulation, we evaluated the effect of Hb on LPS stimulation of peripheral blood mononuclear cell procoagulant activity (i.e., tissue factor, TF). This is another coagulation-based assay for LPS activity which is quantitative (as is the LAL assay) and which is known to correlate well with LPS activity as determined by LAL. A Hb concentration-dependent enhancement of LPS-stimulated procoagulant activity in mononuclear cells was observed (Fig. 14) (30).

Since Hb has the ability to increase the production of TF by mononuclear cells, we reasoned that vascular endothelium might demonstrate a similar response. Cultured human umbilical vein endothelial cell (EC) monolayers were incubated with LPS, in the presence and absence of Hb, and the generation of EC procoagulant activity (TF) was determined. LPS alone (0.01 mg/ml to 10 mg/ml) caused a concentration dependent increase in production of EC TF activity, compared to the TF produced by unstimulated cells. Hb resulted in augmented production of TF in response to LPS (Fig.15)(31). Enhancement was not produced by IgG or human serum albumin (Fig. 16)(32). Enhancement was demonstrated with both native and cross-linked Hbs, and was shown to be

concentration dependent between 0.1 mg/ml and 100 mg/ml Hb. In both the presence and absence of Hb, the production of TF activity by LPS was completely inhibited by Actinomycin D or cycloheximide, indicating a requirement for new protein synthesis. Elevated levels of TF protein in response to Hb-LPS, as assessed by an ELISA assay, also were demonstrated. Inhibition of nitric oxide synthesis, using N-monomethyl-L-arginine (NMMA), resulted in attenuated TF production (10-80% decrease of TF) by the EC in response to both LPS alone and Hb-LPS.

A possible mechanism for the enhancement by Hb of the stimulation of LPS-induced TF production was suggested by the demonstration that Hb increased the binding of LPS to EC (Fig. 17)(32). The increase in binding was related to the concentration of Hb. Furthermore, the increase in binding of LPS was produced only when LPS and Hb had been incubated prior to addition to the EC culture. Increased binding was demonstrable both in serum-containing and serum-free medium, as well as in plasma (Fig. 18). This indicated that soluble CD14 was not necessary for the binding of LPS under the conditions of these experiments. However, in the absence of serum, LPS binding to EC did not produce the biological response characterized by synthesis of TF.

Platelet adherence to endothelial cells. Because of the critical role of the vascular endothelium in promoting pathological hemostatic responses to LPS in vivo (LPS transforms the endothelium from an anticoagulant surface to a procoagulant surface), we also examined whether Hb modified LPS-induced platelet adherence to endothelial cells (EC). Cultured human EC

monolayers were incubated with LPS, in the presence and absence of HbA_0, and the binding of radiolabeled human platelets was examined. LPS alone resulted in slightly increased binding of human platelets to EC in culture (20% increase compared to platelet binding in the absence of LPS), and Hb-LPS complexes further increased platelet binding to EC (35% increase compared to platelet binding in the control without LPS or Hb). Incubation of the EC with Hb alone resulted in a slight decrease in platelet binding.

Complement activation. Enhancement by Hb of the biological activity of LPS in the activation of a proteolytic coagulation cascade in LAL suggested that there may be an impact of Hb on the ability of LPS to activate other protease cascades. We studied whether formation of Hb-LPS complexes altered the ability of LPS to activate and fix complement (a process thought to contribute to the in vivo toxicity of Hb in animal studies). Addition of Hb had little or no effect on the intrinsic complement fixing abilities of eight smooth endotoxins, rough endotoxins, or endotoxin partial structures (33). At higher concentrations (>0.2 mg/ml), Hb by itself also was capable of fixing complement, in the absence of LPS, via the classical pathway of complement activation.

Lethality in Mice. Because of the extensive in vitro data we obtained demonstrating the ability of Hb to enhance the biological activity of LPS, we initiated animal experiments to determine whether LPS-induced mortality was affected by the presence of hemoglobinemia. Mice were injected i.p. with an LD_{50} dose of *E. coli* LPS(500 mg), and 8 hr later received an i.v. infusion of Hb (60

mg) sufficient to raise the blood Hb level by 4 - 5.5 g/dL. LPS-induced mortality was increased at several time points after Hb infusion (Fig. 19). Mortality in the Hb-treated mice was also noted many hours earlier than in mice which had received only LPS. Enhancement of mortality by Hb was observed over a range of doses of injected LPS (34). At a given endotoxin dose, enhancement of mortality was dependent on the dose of Hb administered. In the presence of endotoxemia, doses of Hb ≥45 mg resulted in increased mortality (Table 3). Hb itself caused no mortality, and mice which received Hb alone appeared completely normal throughout the study. Furthermore, Hb increased endotoxin-related mortality in mice whether it was infused intravenously prior to, coincident with, or subsequent to intraperitoneal endotoxin injection (Table 4).

Increased mortality in mice that had received LPS was observed for all preparations of Hb tested, i.e., αα Hb, HbA_0, ßßHb, and ßß/αα Hb (Table 4). This established that the effect of Hb on mortality was not limited to a single preparation of Hb nor was uniquely produced by the nature of the specific chemical crosslink. Other experiments suggested that an increased cytokine response and depressed reticuloendothelial cell function may have contributed to the enhanced mortality from LPS in the presence of Hb. Our observations are consistent with the previous reports that indicate the presence of free Hb in the circulation can compromise reticuloendothelial system function and increase susceptibility to bacterial infection (35-40).

Tumor Necrosis Factor (TNF) Production. Based on the above data that clearance properties of the reticulo-endothelial cell system were altered by the combination of LPS and Hb, we considered the possibility that the responsiveness of the cytokine-producing cells of this system to LPS was modified by the infusion of Hb. Increased production of cytokines by these cells in response to LPS could potentially contribute to enhancement by Hb of LPS-induced mortality. The intravenous infusion of Hb (60 mg/mouse) either prior to or coincident with LPS resulted in peak TNF concentrations in plasma approximately twice those of animals which received only LPS (Fig. 20). This suggested that Hb infusion may have primed TNF-producing cells for subsequent stimulation by LPS. Supporting this hypothesis was the finding that Kupffer cells and peripheral blood mononuclear cells, obtained from Hb-treated mice and then placed in culture, demonstrated increased sensitivity to LPS ex vivo compared to cultured cells from control, untreated mice (Fig. 21) (41). We concluded that LPS-responsive cells became hypersensitive to stimulation by LPS as a result of Hb infusion, and that such a mechanism might contribute to Hb enhancement of mortality in mice. However, the importance of the hypersensitivity of LPS-responsive cells for lethality is not entirely clear, and we (unpublished observations) and others (42,43) have noted that peak plasma TNF levels do not correlate with survival. It has been proposed that localized cytokine production may be of greater importance than circulating plasma cytokines in the pathogenesis of inflammatory disorders such as sepsis (44,45).

Conclusions

Our data strongly support the conclusion that hemoglobin is an endotoxin-binding protein and that as a result, LPS and Hb form complexes. The interaction between LPS and Hb alters each of the components of the Hb-LPS complex (Table 5). Importantly, the biological effects of LPS are enhanced and the UV spectrum of Hb is changed, consistent with methemoglobin formation and denaturation of the Hb molecule. Our observations indicate that the interaction between LPS and Hb results in marked disaggregation of the LPS macromolecule into smaller units that may approximate LPS monomers. The association between disaggregation and an increase in the biological activity of LPS is consistent with recent studies that have emphasized the relationship between the physical state of the LPS and biological activity (46-50). The spatial conformation of Lipid A aggregates (e.g., lamellar vs non-lamellar) may play an important role in increasing the biological activity of LPS in aqueous biological systems. In addition, the relative concentrations of monomeric versus aggregated forms of LPS may also influence biological activity. However, this remains a controversial issue and may depend, in part, upon the concentration of LPS, its solubility, and the biological system utilized (29,51-53).

The interaction between Hb and LPS occurs with native HbA_0, $\alpha\alpha$Hb, or $\alpha\alpha$HbCO, the three forms of Hb that we have investigated in vitro. Furthermore, a wide variety of LPSs have

been shown to interact with Hb, in the systems we have examined. Importantly, we have demonstrated that the administration of Hb also significantly increases the biological activity of LPS in vivo, as manifested by a marked increase in the mortality of mice that received both LPS and either native or cross-linked Hb. Therefore, our observations have potential relevance for the utilization of hemoglobin solutions as substitutes for red blood cells.

The development of non-infectious, non-antigenic stable red blood cell substitutes for human use is of great importance in both civilian and military settings. Products presently under investigation include a variety of derivatized cell-free Hb preparations, perfluorocarbon emulsions, and encapsulated Hb preparations. Safety of red cell substitutes, as well as efficacy, have been identified as critically important by the Center for Biologics Evaluation and Research (54,55). Our data suggest that hemoglobin-based blood substitutes, which are currently undergoing clinical trials (56,57), may intensify the potentially fatal effects of the sepsis syndrome in patients with trauma, infection or hypotension who receive hemoglobin for red blood cell replacement. Others have also recently expressed concern about the potential danger of administration of hemoglobin-based red blood cell substitutes to patients with sepsis, ischemia, or shock (the latter two clinical conditions can predispose to the development of endotoxemia, even if endotoxin is not the precipitating cause of ischemia or hypotension) (13,58,59). Therefore, Hb should be administered to such patients with caution, and thorough serial physiological observations performed

in order to detect any worsening of signs or symptoms that may be attributable to endotoxemia and the sepsis syndrome.

Notes:

Figures 1, 2, 3, 4, 9, 10 and Table 1 are from Kaca, Roth and Levin, 1994. [Ref. #21]

Figures 5, 6 and Table 2 are from Kaca et al., 1995. [Ref. #23]

Figure 7 is from Yoshida, Roth and Levin, 1995. [Ref. #25]

Figures 8 and 11 are from Kaca et al., 1994. [Ref. #24]

Figures 12 and 13 are from Roth, Wong and Hamilton, 1996. [Ref. #28]

Figure 14 is from Roth et al., 1993. [Ref. #30]

Figure 15 is from Roth, 1994. [Ref. #31]

Figures 16, 17 and 18 are from Roth, 1996. [Ref. #32]

Figures 20 and 21 are from Su et al, 1999 [Ref. #41]

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APPENDIX (LEGENDS, TABLES, AND FIGURES)

FIGURES: 20

TABLES: 5

Figure Legends

Figure 1. Binding of LPS to immobilized Hb. αα Hb (1 mg/well) was immobilized in microtiter plate wells, and ^{125}I-LPS was added. Bound LPS was determined by gamma counting, and specific binding was calculated by subtracting bound ^{125}I-LPS in wells without Hb. (From Ref. 21.)

Figure 2. Photoaffinity labeling of Hb with ^{125}I-LPS-ASD. ^{125}I-LPS-ASD was incubated with αα Hb, photolyzed with UV light, and electrophoresed in SDS and 2-mercaptoethanol. Following electrophoresis, the gel was stained with Coomassie blue (A, left lane), dried, and subjected to autoradiography (A, right lane). Another photoaffinity-labeled αα Hb preparation from a separate experiment is shown (B, left lane), along with controls that consisted of an incubation mixture containing 100-fold excess unlabeled LPS as a blocking agent to demonstrate inhibition of specific binding (B, middle lane) and ^{125}I-LPS-ASD alone (B, right lane). (From Ref. 21.)

Figure 3. Sucrose density centrifugation of LPS-Hb. ^{14}C-LPS was incubated with αα Hb (100 mg/ml), and the mixture centrifuged through a 4-20% continuous sucrose gradient. 0.4 ml fractions were assayed for hemoglobin by absorbance at 405 nm (closed symbols), and for LPS by scintillation counting (open symbols). (From Ref. 21.)

Fig. 4. Electrophoresis of LPS and Hb. ^{14}C-LPS was incubated with ααHb, and the ααHb-LPS mixture or LPS alone was electrophoresed in polyacrylamide in the absence of SDS. ^{14}C-LPS was measured by scintillation counting of gel pieces (closed symbols), and ααHb was monitored by absorbance at 405 nm (open circles). (From Ref. 21.)

Fig. 5. Time-dependent conversion of ααHb (A) and HbA_0 (B) to metHb and hemichromes in the presence of *S. minnesota* 595 OH37 LPS (0.3 mg/ml and 0.8 mg/ml LPS incubated with ααHb and HbA_0, respectively). Percentages of oxyHb, metHb, and hemichromes were determined according to the method of Winterbourn(Winterbourn, C.C. Methods Enzymol 1990; 186: 256-274.). Open symbols, Hb alone; closed symbols, Hb + LPS. (From Ref. 23.)

Fig. 6. Time course of changes in the hemoglobin absorption spectrum in the presence of LPS. ααHb (21 mM in PBS, pH 7.4) was incubated at 37°C in the presence of 0.3 mg/ml *S. minnesota* 595 OH37 LPS, and absorbance spectra in the Soret (A) and visible (B) regions of the Hb spectrum were obtained at various times of incubation. 1 - initial spectrum of ααHb alone; 2 - 10 min; 3 - 20 min; 4 - 40 min; 5 - 90 min; 6 - 120 min. The sample cuvette contained Hb in PBS with or without LPS, and the reference cuvette contained PBS (for ααHb spectra alone) or LPS alone (0.3 mg/ml in PBS) (for ααHb-LPS mixture spectra). The arrows indicate the apparent isosbestic points. (From Ref. 23.)

Figure 7. Intravascular clearance of ^{125}I-LPS after intravenous injection into rabbits: LPS only (E), LPS administered immediately following a 10 minute infusion of HSA (C), or LPS following hemoglobin (Hb)(G). The numbers of animals in each group were 6, 6, and 5, respectively. Values represent the mean percent of the level of radioactive LPS in whole blood at $T_0 \pm$ SD. (From Ref. 25.)

Fig. 8. Enhancement by hemoglobin of the activation of Limulus amebocyte lysate (LAL) by Proteus LPS. LAL reactivities of LPS (500 ng/ml) in the absence (-Hb) or presence (+Hb) of αα cross-linked hemoglobin (1 mg/ml) were determined with the chromogenic LAL assay. To determine relative LAL activities, a standard curve of *P. mirabilis* R45 LPS was prepared, which related absorbance to LPS concentration. Using this standard curve, the absorbance for each sample (LPS alone or LPS-Hb) was converted into the equivalent R45 LPS concentration. 500 ng/ml R45 LPS was assigned a relative LAL activity of 1. Samples were assayed with eight replicates, and results are expressed as the mean ± 1 SD. (From Ref. 24.)

Fig. 9. Enhancement of LPS activation of Limulus amebocyte lysate (LAL) by Hb. Dilutions of *E. coli* O26:B6 LPS (A) or *P. mirabilis* S1959 LPS (B) in ααHbCO (◆), ααHb (■), HbA_0 (●), HSA (▼) or NaCl (O), were assayed with the chromogenic LAL test. Absorbances at 405 nm were measured at 5 min. All protein concentrations were 1 mg/ml. Incubations were performed in triplicate, and the mean is shown. (From Ref. 21.)

Fig. 10. Enhancement of LPS activation of Limulus amebocyte lysate (LAL) by Hb. Mixtures of LPS (100 pg/ml *E. coli* O26:B6) and HbA_0 (O), ααHbCO (●), ααHb (Δ) or HSA (◆) were assayed with LAL, using gelation as the endpoint. Protein concentrations ranged from 0.01-2 mg/ml. Enhancement of activation of LAL was calculated by comparison of the gelation time of each mixture to the gelation times for LPS solutions in 0.9% NaCl. 100 pg/ml LPS in 0.9% NaCl solution gelled Limulus amebocyte lysate in 2.5 hr. Similar results were obtained from each of three independent experiments. (From Ref. 21.)

Fig. 11. Turbidity and biological activity of LPS in the absence and presence of Hb. Various concentrations of αα cross-linked Hb (from 0.01 to 1.0 mg/ml) were added to LPS (final concentration, 1 mg/ml) in microtiter plate wells and absorbances were measured at 620 nm. The turbidity of each LPS (absorbance at 620 nm) in the absence of Hb has been designated as 0, and the change in absorbance induced by Hb is shown. Absorbances due to Hb have been subtracted. Actual baseline LPS absorbances were as follows: *P. mirabilis* R110, 0.21; *S. minnesota* R595, 0.12. LAL then was added to each well and chromogenic activities determined at 405 nm. (From Ref. 24.)

Fig. 12. Top: Highly aggregated *S. minnesota* (Re) 595 LPS demonstrating primarily ribbon-like (arrows) and mesh-like (open arrows) structures (x60,000). Middle: Highly aggregated *S. minnesota* (Re) 595 LPS demonstrating primarily large membrane-like sheets (arrow heads) (x60,000). Bottom: High-power image (x180,000) of

ribbon-like (arrows) and mesh-like (open arrow) Re LPS structures. (From Ref. 28.)

Fig. 13. Top: Hb alone, consisting of uniform particles ~5.5 nm (x180,000). Bottom: Hb plus *S. minnesota* (Re) 595 LPS, following incubation at 37°C for 18 hr, showing disaggregation of LPS into small disc (arrow) and lens-shaped (arrow head) particles of 5-20 nm (x180,000). None of the ribbon-like, mesh-like or membrane-like structures of LPS remain after incubation with Hb. Very small Hb-LPS complexes are not distinguishable from Hb alone. (From Ref. 28.)

Fig. 14. Tissue factor (TF) production by human mononuclear cells. Human mixed mononuclear cells were incubated with LPS in the presence of various concentrations of endotoxin-free Hb(0.6-60 mg/ml). TF generated by LPS alone and the Hb-LPS mixtures was determined following addition of citrated plasma and calcium(plasma recalcification assay). The contribution of the Hb alone(at each concentration, respectively) to the total TF generated by the mononuclear cells was subtracted from the measured total. (From Ref. 30.)

Fig. 15. Effect of Hb on the production of human umbilical vein endothelial cell tissue factor (TF) in response to LPS. Cultured human endothelial cells (EC) were incubated with ααHb alone, LPS alone, or LPS in the presence of ααHb. TF actvities then were determined with the plasma recalcification assay. (From Ref. 31.)

Fig. 16. Effect of proteins on endothelial cell tissue factor (TF) activity. LPS was preincubated with Hb, HSA or IgG, and LPS alone or the LPS-protein mixtures then were added to human umbilical vein endothelial cells (EC) in medium containing 4% bovine serum. After 1 hr, the EC were washed, freeze-thawed and sonicated, and the plasma recalcification assay for TF performed. TF activity was assessed by the rate of increase in absorbance at 340 nm. Mean ± SD absorbances from 12 replicate wells are presented. (From Ref. 32.)
* $p < .01$ increase vs. LPS alone (Student's T-test).

Fig. 17. Effect of Hb on binding of LPS to human umbilical vein endothelial cells (HUVEC). ^{3}H-LPS was preincubated with various concentrations of Hb, and ^{3}H-LPS alone or the LPS-Hb mixtures then were added to HUVEC in medium containing 4% bovine serum. After 30 min, bound LPS was determined by scintillation counting. Binding assays were performed in two replicate wells per condition. Means and ranges are presented. (From Ref. 32.)
* $p < .01$ increase vs. LPS alone (Student's T-test).

Fig. 18. Effect of proteins on binding of LPS to human umbilical vein endothelial cells (HUVEC). ^{3}H-LPS was preincubated with Hb, HSA or IgG (each 10 mg/ml, final concentration), and ^{3}H-LPS alone or the LPS-protein mixtures then were added to HUVEC. After 30 min, bound LPS was determined by scintillation counting. In some wells, ^{3}H-LPS was added to the medium first, followed by the subsequent addition of Hb 5 min later. Binding assays were performed, with 6

replicate wells, in complete medium containing 4% bovine serum (A), in serum-free medium (B) or in 100% plasma (anticoagulated with hirudin) in the absence of medium (C). Means ± SD are presented. (From Ref. 32.)

Fig. 19. Effect of Hb on mortality from LPS. Mice were injected i.p. with an LD_{50} dose of LPS(500 mg/animal), followed 8 hr later by i.v. infusion of 60/mg Hb per mouse (which generated a peak plasma Hb concentration of 4.0 - 5.5 g/dL) or 0.9% NaCl. Survival for 30 hr after Hb infusion is shown.

Fig. 20. Effect of Hb on induction of plasma Tumor Necrosis Factor (TNF) by LPS. Top: Mice were injected intravenously with Hb (60 mg/mouse) or NaCl, and 10 hr later were injected intraperitoneally with an LD50 dose of *E. coli* LPS (500 mg). Plasma TNF concentrations following LPS administration were determined by ELISA. TNF levels (mean ± SE) of 35 mice (Hb + LPS) and 20 mice (NaCl + LPS), respectively, are shown. Bottom: Mice were injected intraperitoneally with an LD50 dose of LPS (500 mg), and then immediately were injected intravenously with Hb (60 mg/mouse) or NaCl. Plasma TNF concentrations following LPS administration were determined by ELISA. TNF levels (mean ± SE) of 16 mice (LPS + Hb) and 15 mice (LPS + NaCl), respectively, are shown. * $p<.05$ (Mann-Whitney U test). (From Ref. 41.)

Fig. 21. Tumor Necrosis Factor (TNF) production by LPS-stimulated Kupffer cells and peripheral blood mononuclear cells (PBMC) obtained from Hb-infused or control mice. Top: Mice were injected intravenously with Hb (60 mg/mouse) or NaCl. Ten hr later, pronase was injected, the liver was excised and digested with DNase and pronase, and the digested liver preparations were centrifuged on Accudenz to isolate Kupffer cells. The Kupffer cell-enriched preparations were placed in culture and stimulated with LPS for 5 hr; TNF in the culture medium was measured by ELISA. Six independent experiments were performed and the results pooled. TNF concentrations are shown (mean $\pm$ SE of 17-23 measurements at each LPS concentration). * $p<.05$ (Mann-Whitney U test). The Hb-treated and control groups also were significantly different by repeated measures ANOVA ($p<.05$).

Bottom: Mice were injected intravenously with Hb (60 mg/mouse) or NaCl. Ten hr later, PBMC were isolated, placed in culture, and stimulated with LPS for 5 hr; TNF in the culture medium was measured by ELISA. Monocytes comprised 3-7% of total PBMC. Four independent experiments were performed and the results pooled. TNF concentrations are shown (mean $\pm$ SE of 8 measurements at each LPS concentration). PBMC from Hb-treated mice appeared to generate more TNF in response to LPS, ex vivo, than control mice, but the differences were not statistically significant. (From Ref. 41.)

Table 1. Ultrafiltration of *E. coli* O26:B6 and *P. mirabilis* S1959 LPS, Hb, and LPS-Hb mixtures*

	E. coli LPS filtered (%)	*P. mirabilis* LPS filtered (%)	
	300 kDa** filter	100 kDa** filter	300 kDa** filter
LPS alone	10.2 ± 2.3	0	15.6 ± 5.6
ααHb alone	0***	0	0
ααHb + LPS	87.3 ± 8.0	63.6 ± 18.7	97.1 ± 1.5
ααHbCO alone	0	0	0
ααHbCO + LPS	89.3 ± 1.5	71.1 ± 4.0	90.9 ± 4.5
HbA_0 alone	0	0	0
HbA_0 + LP	88.1 ± 3.7	71.6 ± 8.8	93.5 ± 8.6

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* Each experiment was performed three times and the mean ± 1 SD is shown. Percent of LPS filtered was determined with the chromogenic LAL test. LPS was quantified with reference to standard curves consisting of the respective LPS/protein mixture prior to filtration.

** Molecular weight cut-off of the filter.

*** Lack of detectable LPS indicates that the starting preparations of Hb were endotoxin-free.

Source: Ref. 21.

Table 2. P_{50} values for Hb and Hb-LPS complexes*

	P_{50}
ααHb alone	26.6
ααHb + LPS[a]	25.1
ααHb + LPS[b]	25.6
HbA_0 alone	9.6
HbA_0 + LPS[c]	8.7
HbA_0 + LPS[d]	7.3

*Oxygen affinity measurements were obtained for cross-linked (ααHb) and native (HbA_0) hemoglobins alone or in the presence of LPS after a hr incubation at 37°C. Measurements were obtained prior to the production of oxidized Hb species. P_{50} was determined utilizing both smooth and rough LPSs: [a] *P. mirabilis* 03 (smooth) LPS; [b] *S. minnesota* Re 595 (rough) LPS; [c] *E. coli* 026 (smooth) LPS; [d] *S. minnesota* 595 OH37 (rough) LPS. Equal concentrations of Hb and LPS were utilized (each at 1 mg/ml prior to dilution in Hemox buffer).

Source: Ref. 23.

Table 3. LPS lethality in mice, after the administration of various doses of ααHb*

	Survival at 48 hr (%)
LPS alone	59
LPS + 6 mg Hb	60
LPS + 11 mg Hb	60
LPS + 22 mg Hb	50
LPS + 45 mg Hb	12**
LPS + 60 mg Hb	7**

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*Swiss Webster female mice (28-32 g) were injected intraperitoneally with 0.5 mg LPS (*E. coli* 055:B5 LPS, in sterile, pyrogen-free saline). 8-10 hr following LPS injection, the mice were infused by tail vein with either 0.6-0.8 ml saline or ααHb in Ringer's acetate, pH 7.4 (doses of Hb ranged from 6-60 mg/mouse). Survival was monitored at 48 hr.

** $p<.01$ vs. LPS alone (Fisher's exact P value).

Table 4. LPS lethality in mice, in the absence and presence of αα Hb, ββ Hb, HbA_0 or HSA*

	Survival at 24 hr (%)	Survival at 48 hr (%)
LPS alone (n=77)	96	55
αα Hb alone (n=10)	100	100
αα Hb + LPS (Hb before LPS) (n=11)	54**	36
αα Hb + LPS (Hb with LPS) (n=28)	32**	7**
αα Hb + LPS (Hb after LPS) (n=56)	48**	7**
HbA_0 alone (n=14)	100	100
HbA_0 + LPS (Hb with LPS) (n=19)	32**	0**
ββ Hb + LPS (Hb after LPS) (n=23)	78**	9**

ββ/ααHb + LPS (Hb after LPS) (n=23)	74**	9**
HSA + LPS (HSA with LPS) (n=27)	100	48
HSA + LPS (HSA after LPS) (n=10)	90	30

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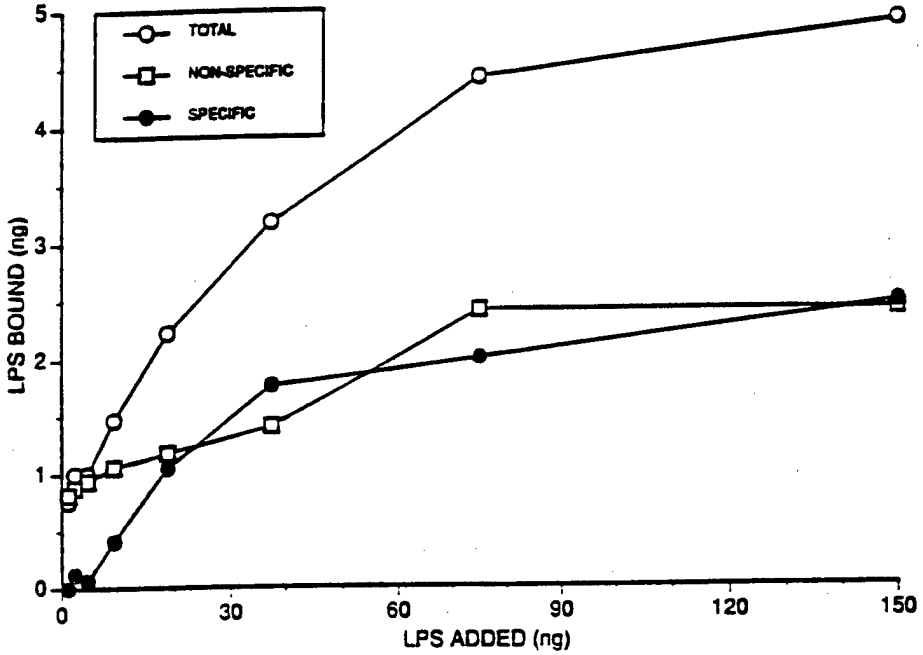
*Swiss Webster female mice (28-32 g) were injected intraperitoneally with saline or with 0.5 mg LPS (*E. coli* 055:B5 LPS, in sterile, pyrogen-free saline). Mice also were infused by tail vein with either 0.6-0.8 ml saline, Hb solutions in Ringer's acetate, pH 7.4 (60 mg/mouse), or human serum albumin (HSA, 60 mg/ml, pH 7.4, in saline with sodium bicarbonate). In various experiments, human ααHb(DBBF cross-linked) was infused either 12 hr prior to, coincident with, or 8-10 hr subsequent to LPS. Bovine fumaryl ßß cross-linked Hb and human deca-sebacyl ßß/αα cross-linked Hb were infused 8-10 hr subsequent to LPS, and HbA_0 was infused coincident with LPS. HSA was infused either coincident with or 8-10 hr subsequent to LPS. Survival was monitored at 24 and 48 hr.

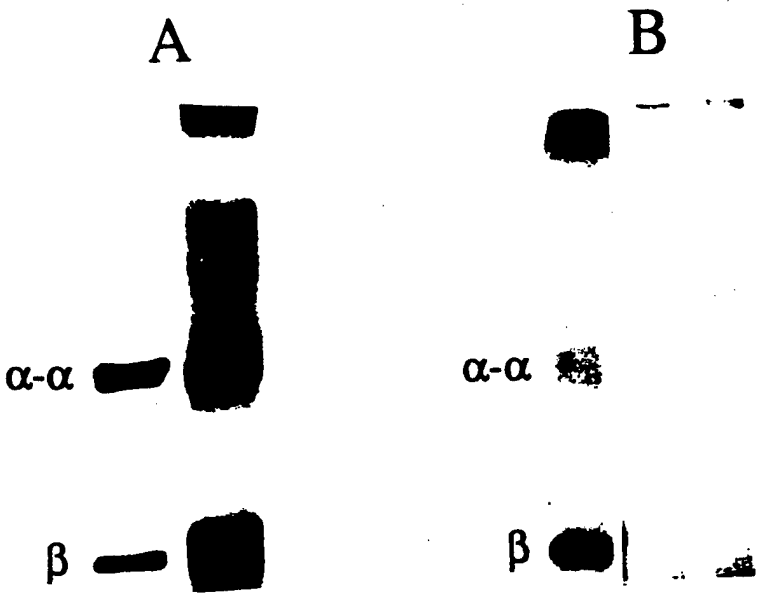
** $p<.01$ vs. LPS alone (Fisher's exact P value).

Table 5. Alterations of hemoglobin (Hb) or bacterial endotoxin (LPS) following their interaction

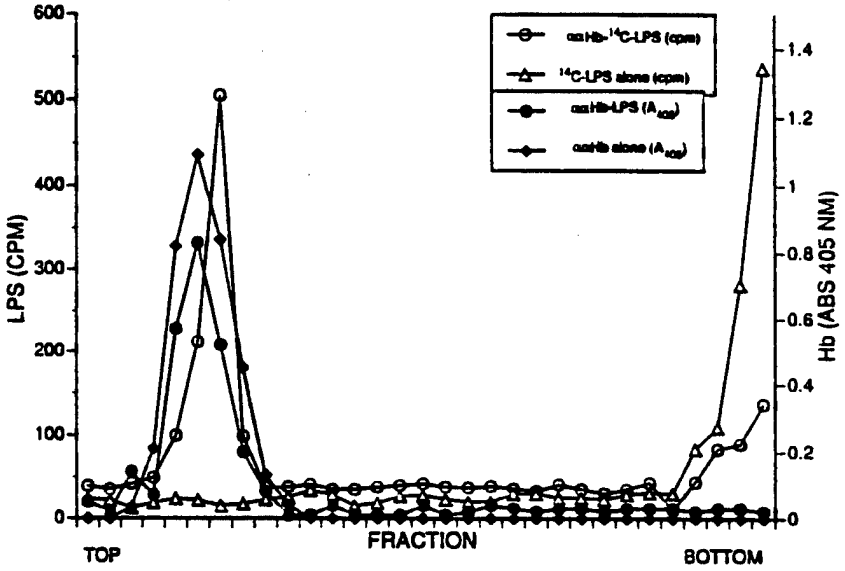
1. MW of LPS markedly decreased
2. Density of LPS decreased
3. LPS and Hb co-electrophoresed
4. Ethanol precipitability of Hb increased
5. Biological effects of LPS enhanced
6. Visible spectrum of Hb altered
   (MetHb and/or hemichromes formed)


TOTAL
NON-SPECIFIC
SPECIFIC
LPS BOUND (ng)
LPS ADDED (ng)
0
1
2
3
4
5
0
30
60
90
120
150

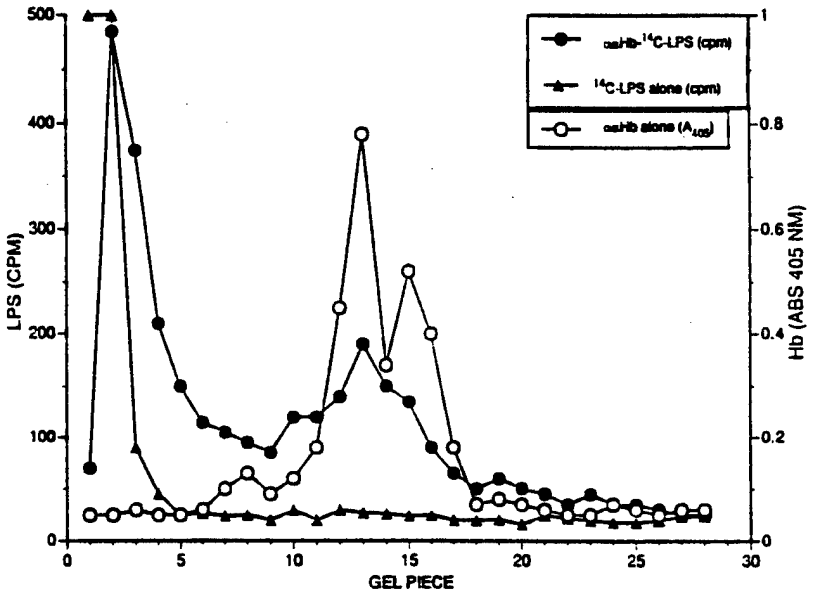
LEVIN
Fig. 1



LEVIN
Fig. 2


600
500
400
300
200
100
0
LPS (CPM)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Hb (ABS 405 NM)
TOP
FRACTION
BOTTOM

LEVIN
Fig. 3


LPS (CPM)
Hb (ABS 405 NM)
GEL PIECE
0
100
200
300
400
500
0.2
0.4
0.6
0.8
1
0
5
10
15
20
25
30

LEVIN
Fig. 4

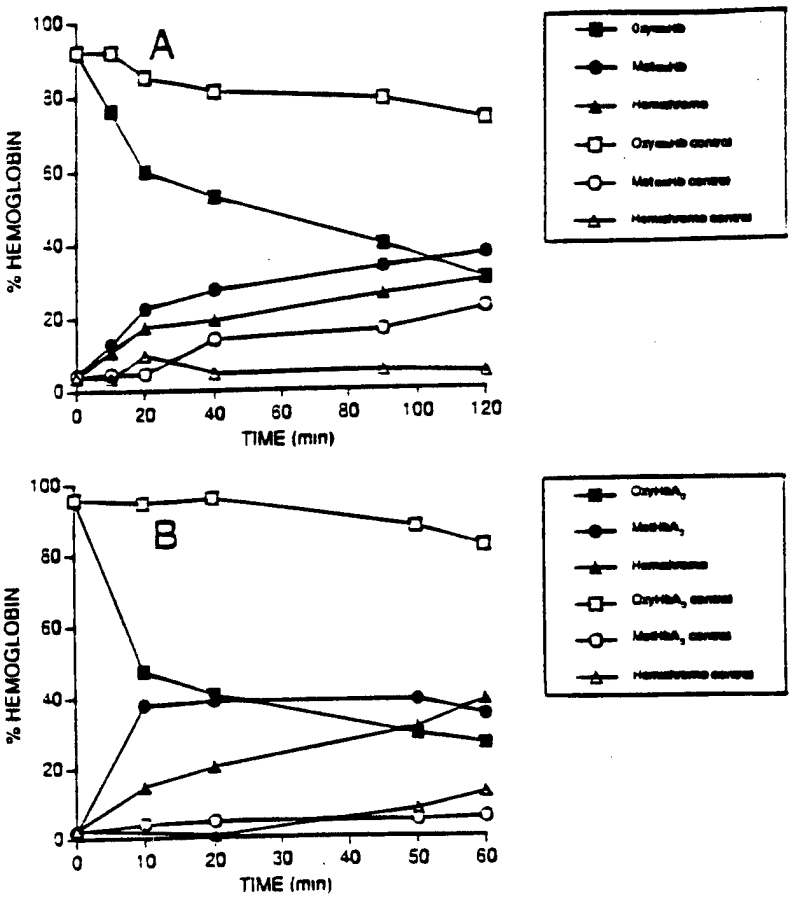


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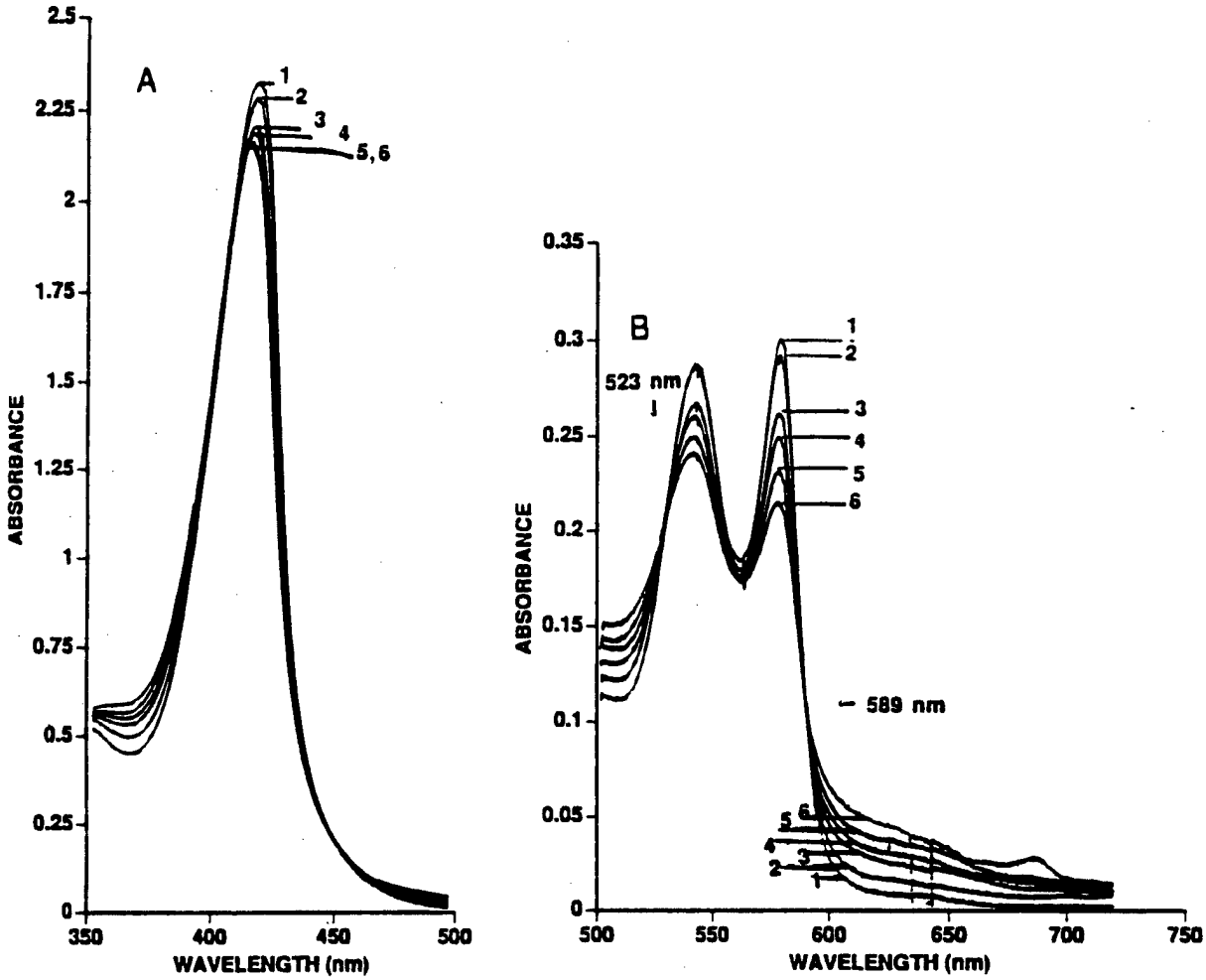
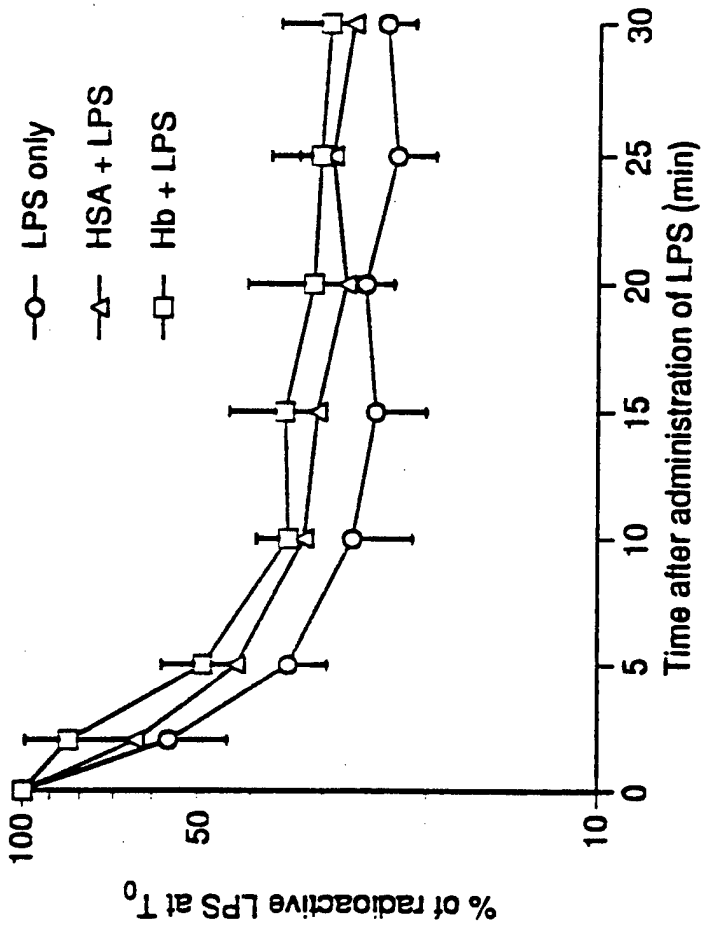
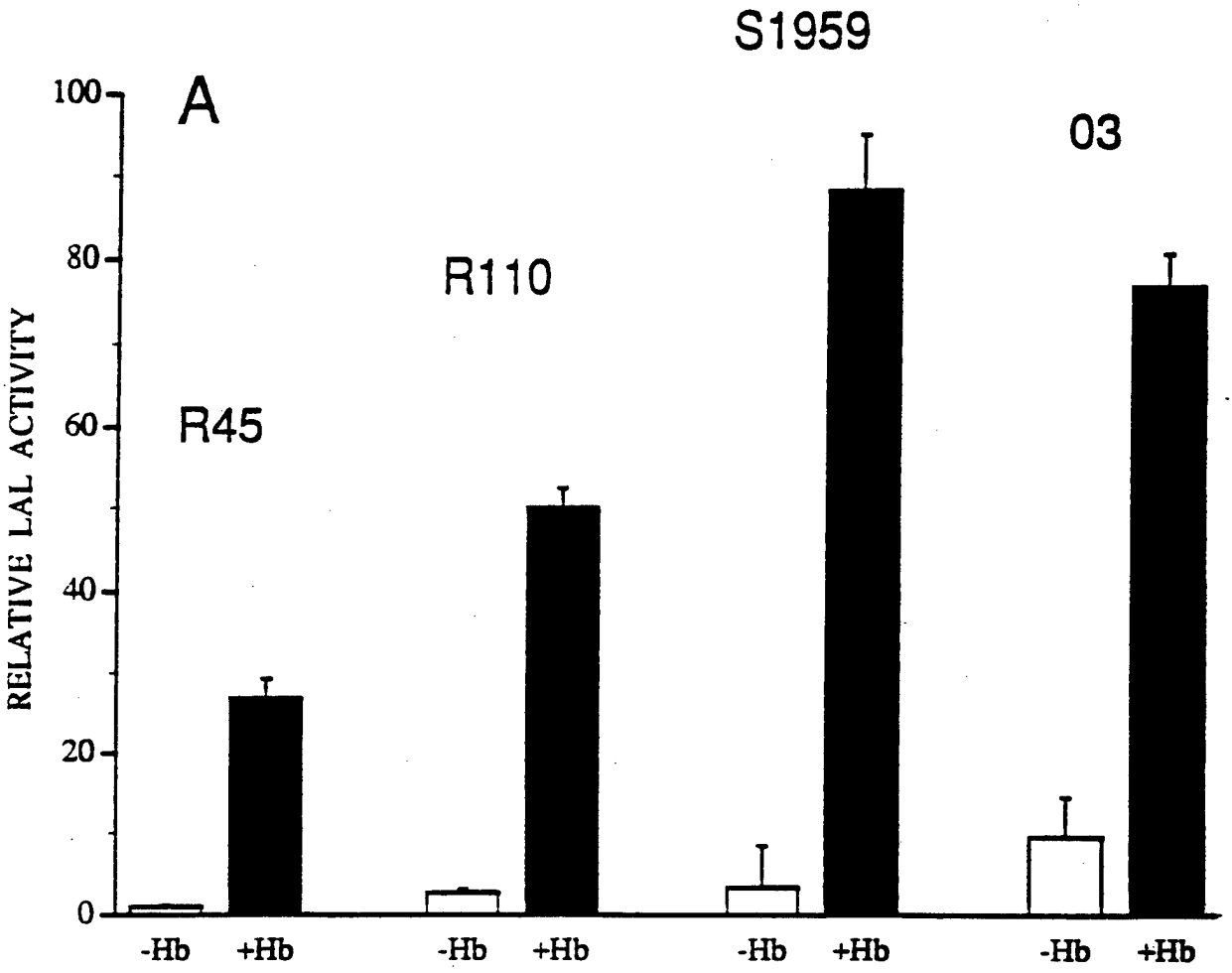
Fig. 5



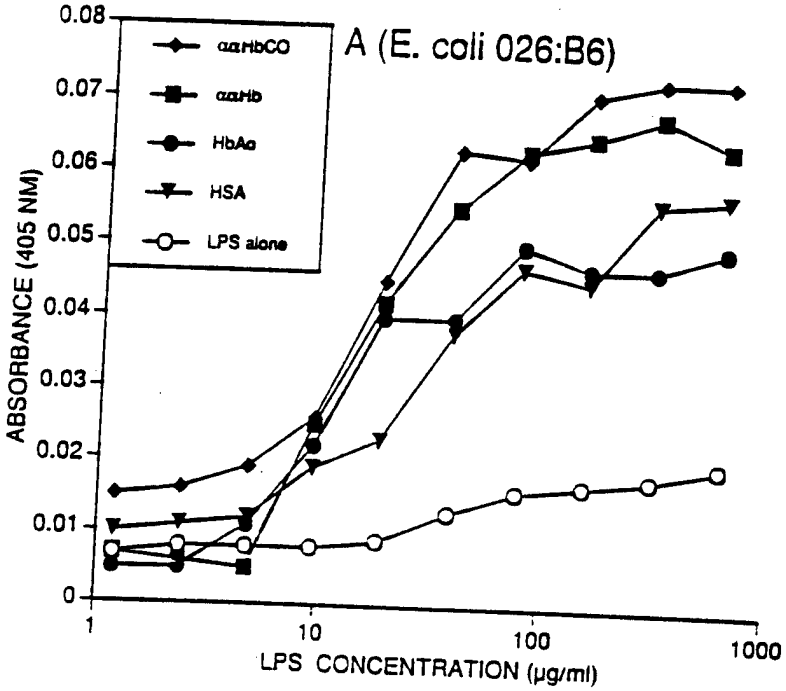
LEVIN
Fig. 6

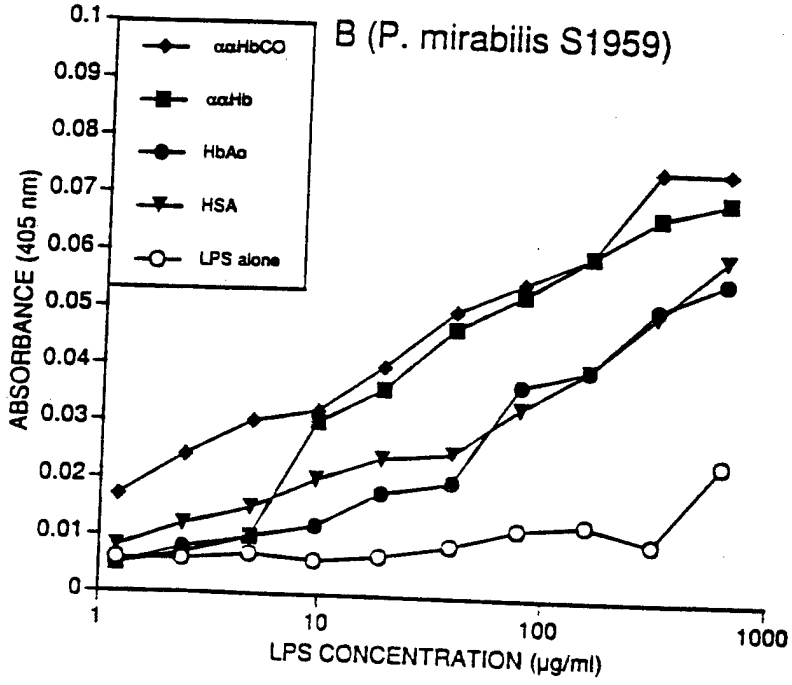

LPS only
HSA + LPS
Hb + LPS
100
50
10
0
5
10
15
20
25
30
Time after administration of LPS (min)
% of radioactive LPS at T_0

LEVIN
Fig. 7


S1959
A
03
R110
R45
RELATIVE LAL ACTIVITY
100
80
60
40
20
0
-Hb
+Hb
-Hb
+Hb
-Hb
+Hb
-Hb
+Hb

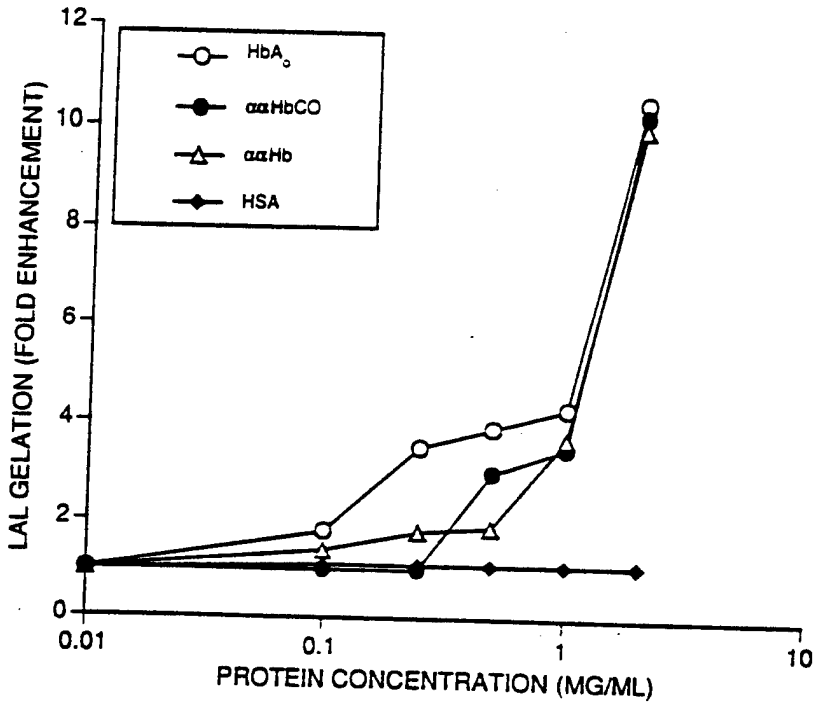
LEVIN
Fig. 8


A (E. coli 026:B6)
αα HbCO
αα Hb
HbAo
HSA
LPS alone
ABSORBANCE (405 NM)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
1
10
100
1000
LPS CONCENTRATION (µg/ml)

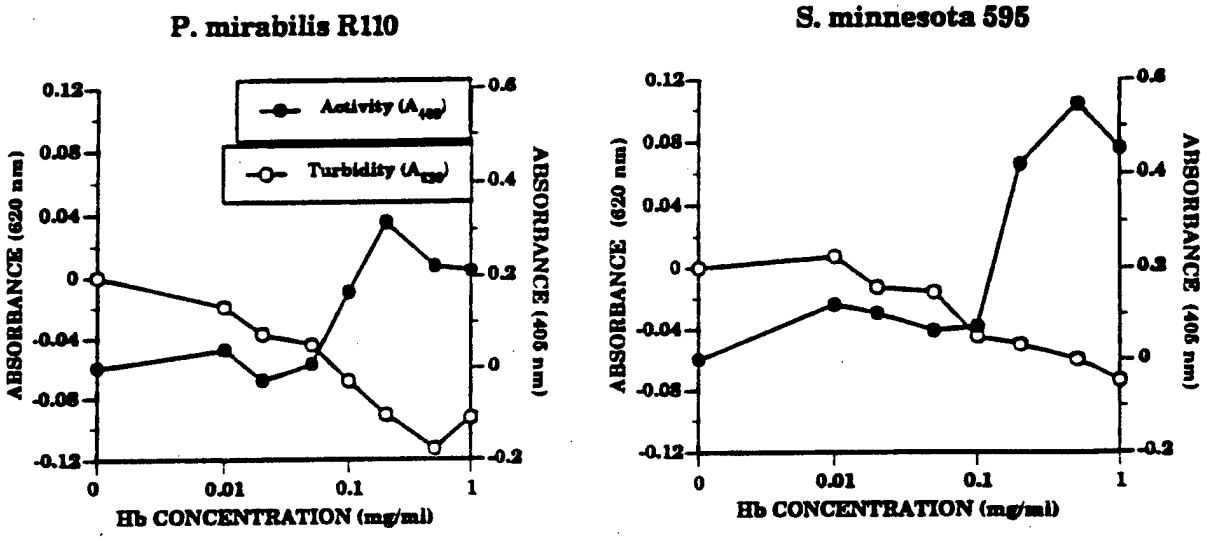

B (P. mirabilis S1959)
αα HbCO
αα Hb
HbAo
HSA
LPS alone
ABSORBANCE (405 nm)
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
1
10
100
1000
LPS CONCENTRATION (µg/ml)

LEVIN

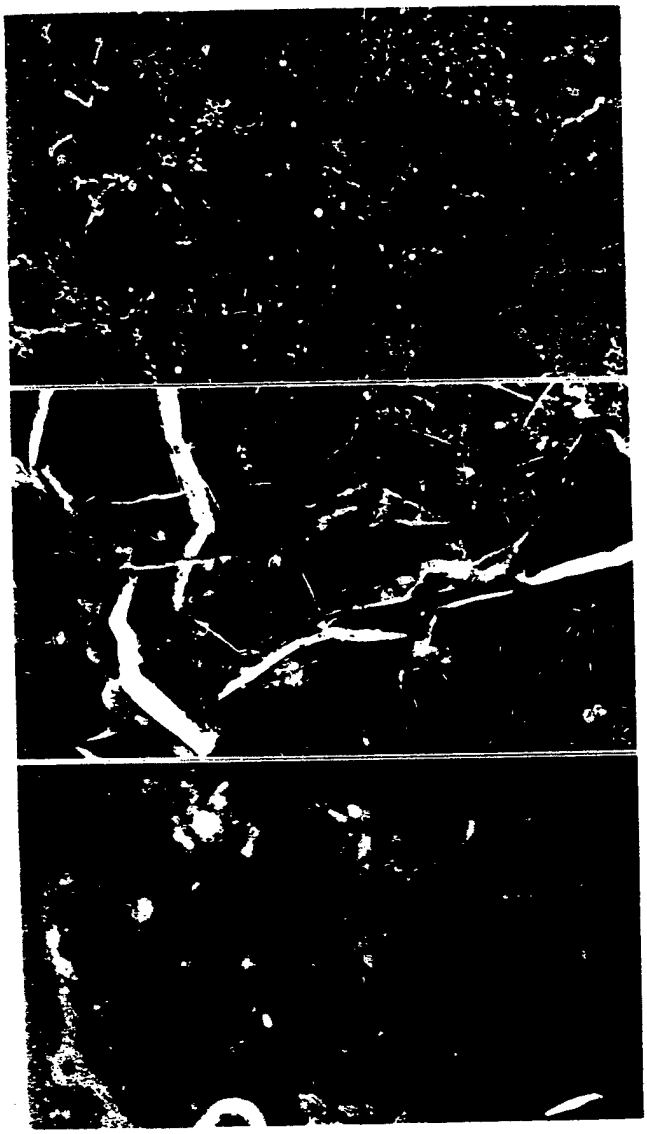
Fig. 9


12
10
8
6
4
2
0
LAL GELATION (FOLD ENHANCEMENT)
HbA$_0$
αα HbCO
αα Hb
HSA
0.01
0.1
1
10
PROTEIN CONCENTRATION (MG/ML)

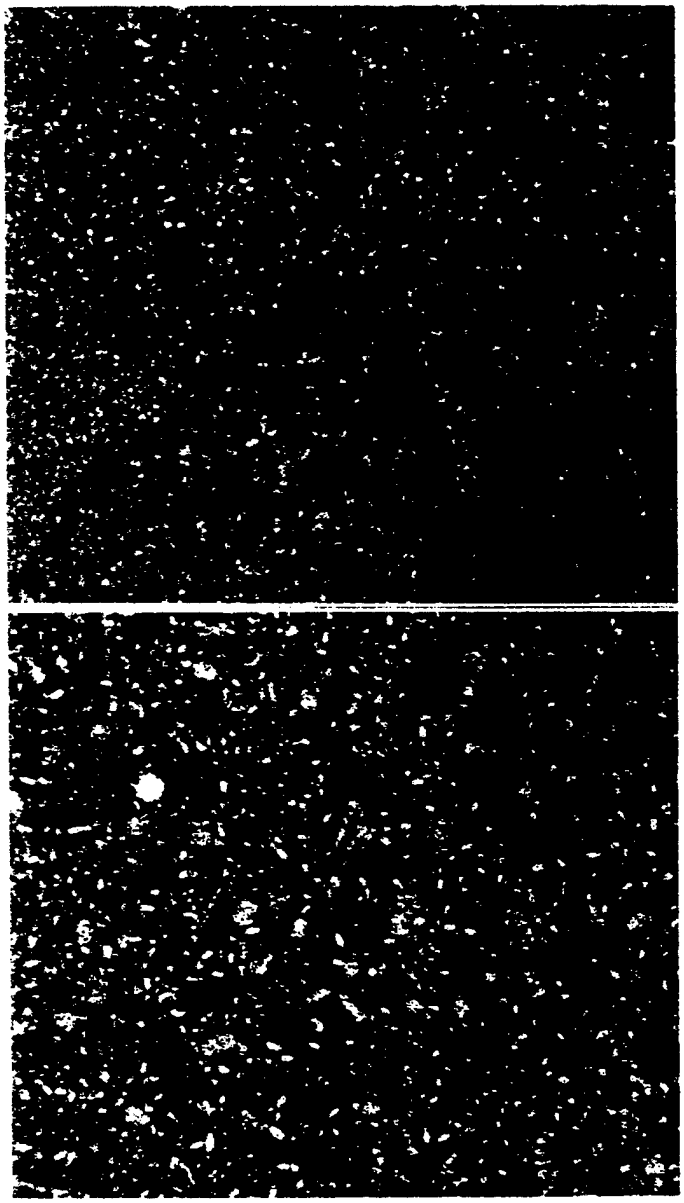
LEVIN
Fig. 10


P. mirabilis R110
S. minnesota 595
Activity (A_{405})
Turbidity (A_{620})
ABSORBANCE (620 nm)
ABSORBANCE (405 nm)
0.12
0.08
0.04
0
-0.04
-0.08
-0.12
0.6
0.4
0.2
0
-0.2
0
0.01
0.1
1
Hb CONCENTRATION (mg/ml)

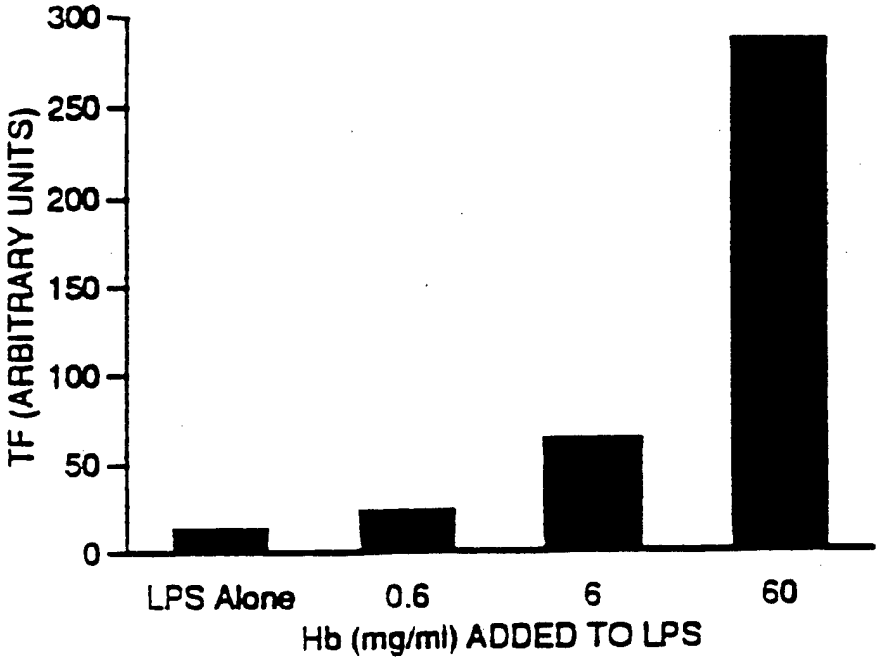
LEVIN
Fig. 11



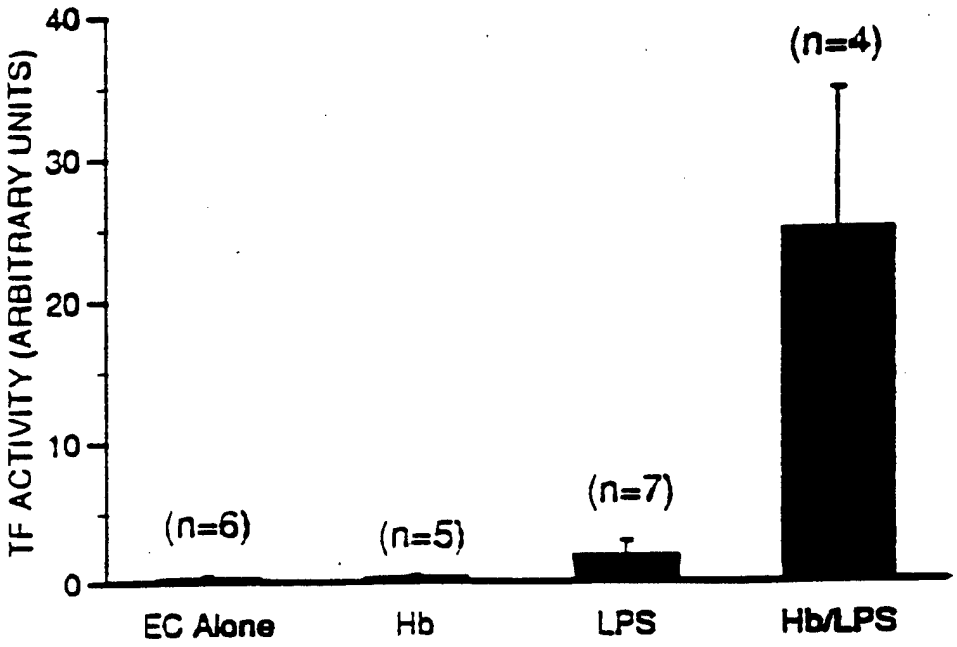
LEVIN
Fig. 12



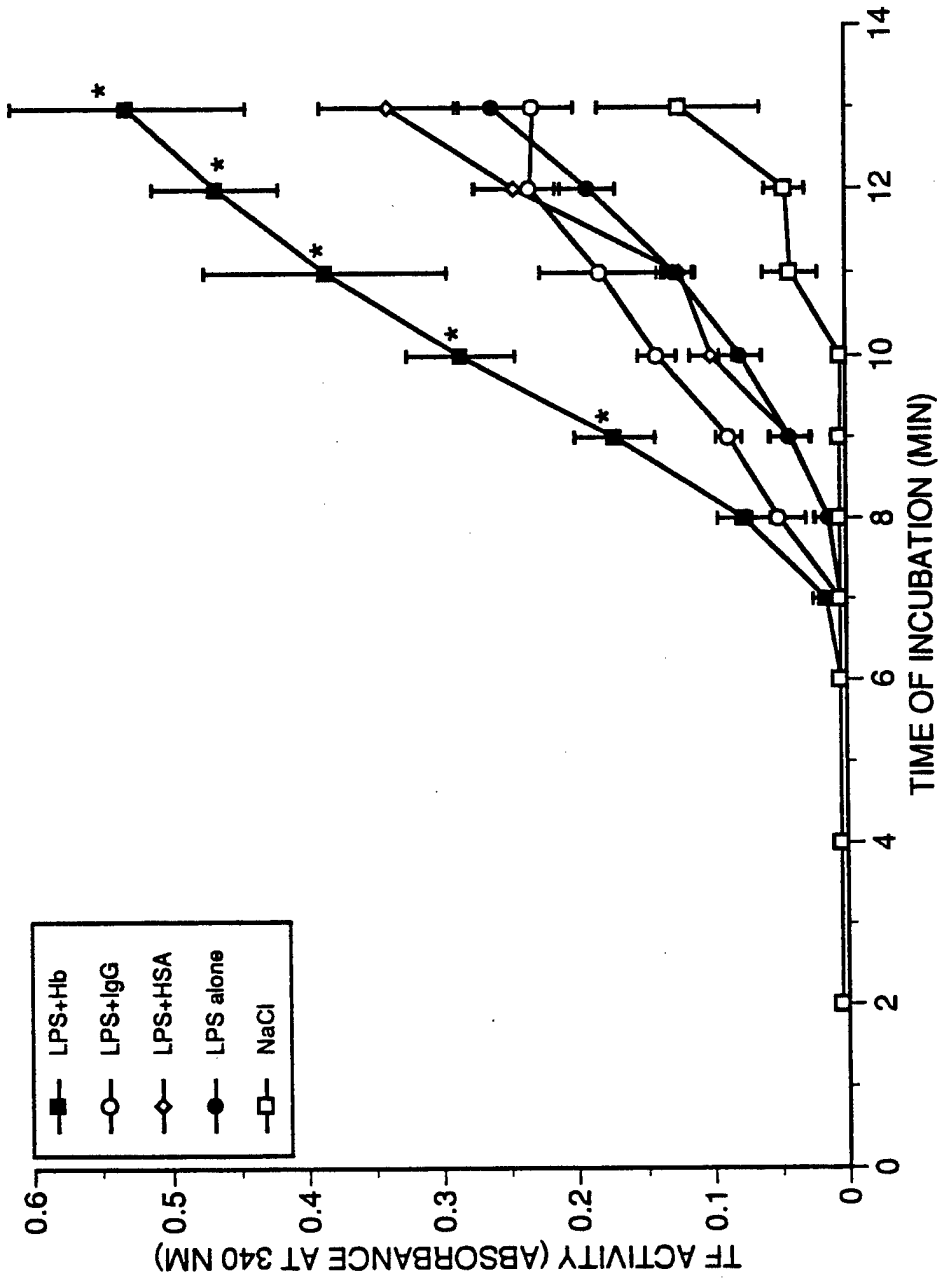
LEVIN
Fig. 13


300
250
200
150
100
50
0
TF (ARBITRARY UNITS)
LPS Alone
0.6
6
60
Hb (mg/ml) ADDED TO LPS

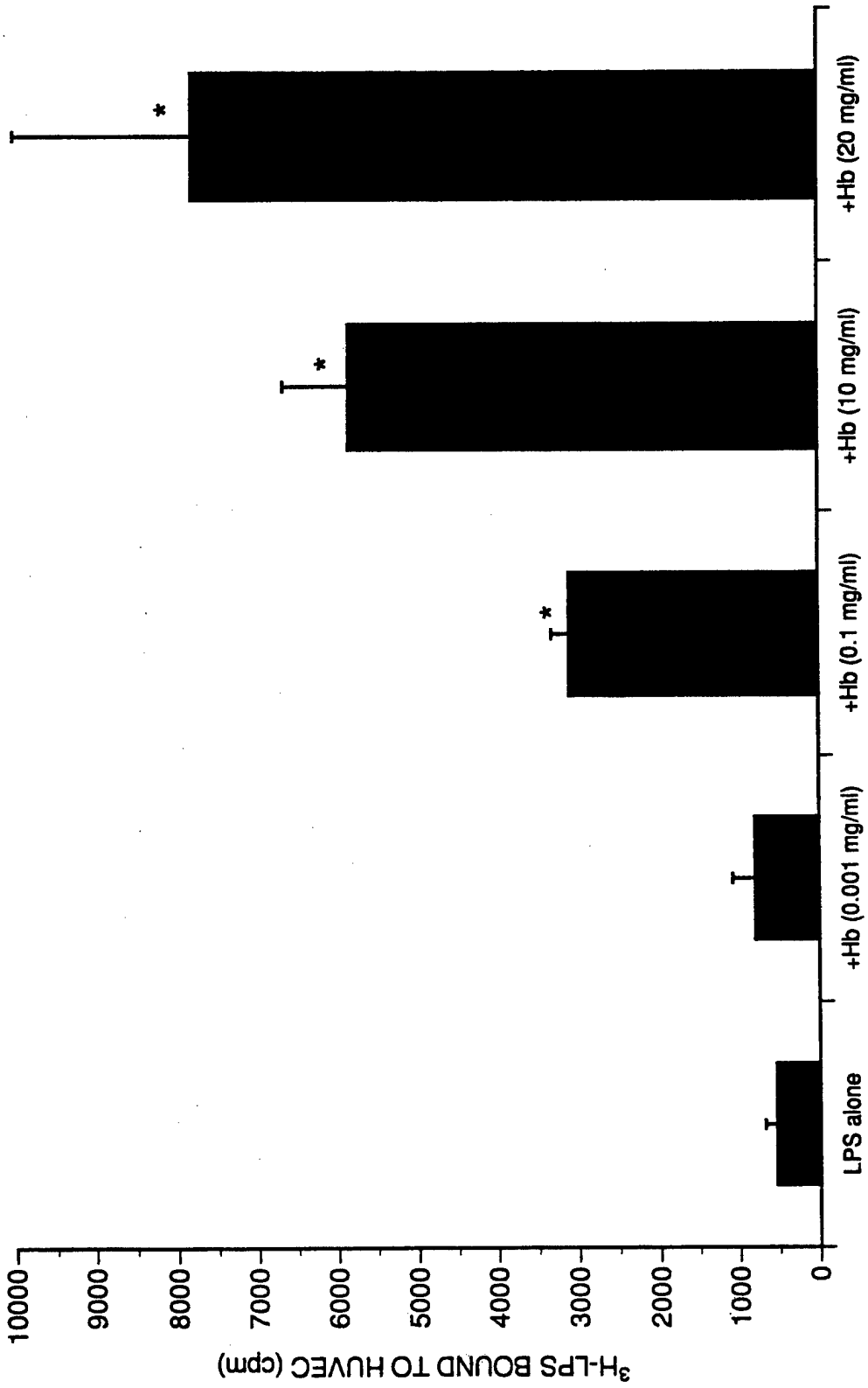
LEVIN
Fig. 14


TF ACTIVITY (ARBITRARY UNITS)
40
30
20
10
0
(n=6)
(n=5)
(n=7)
(n=4)
EC Alone
Hb
LPS
Hb/LPS

LEVIN
Fig. 15


TF ACTIVITY (ABSORBANCE AT 340 NM)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
2
4
6
8
10
12
14
TIME OF INCUBATION (MIN)
LPS+Hb
LPS+IgG
LPS+HSA
LPS alone
NaCl
*
*
*
*
*

LEVIN
Fig. 16


^{3}H-LPS BOUND TO HUVEC (cpm)
0
1000
2000
3000
4000
5000
6000
7000
8000
9000
10000
LPS alone
+Hb (0.001 mg/ml)
+Hb (0.1 mg/ml)
+Hb (10 mg/ml)
+Hb (20 mg/ml)
*
*
*

LEVIN
Fig. 17

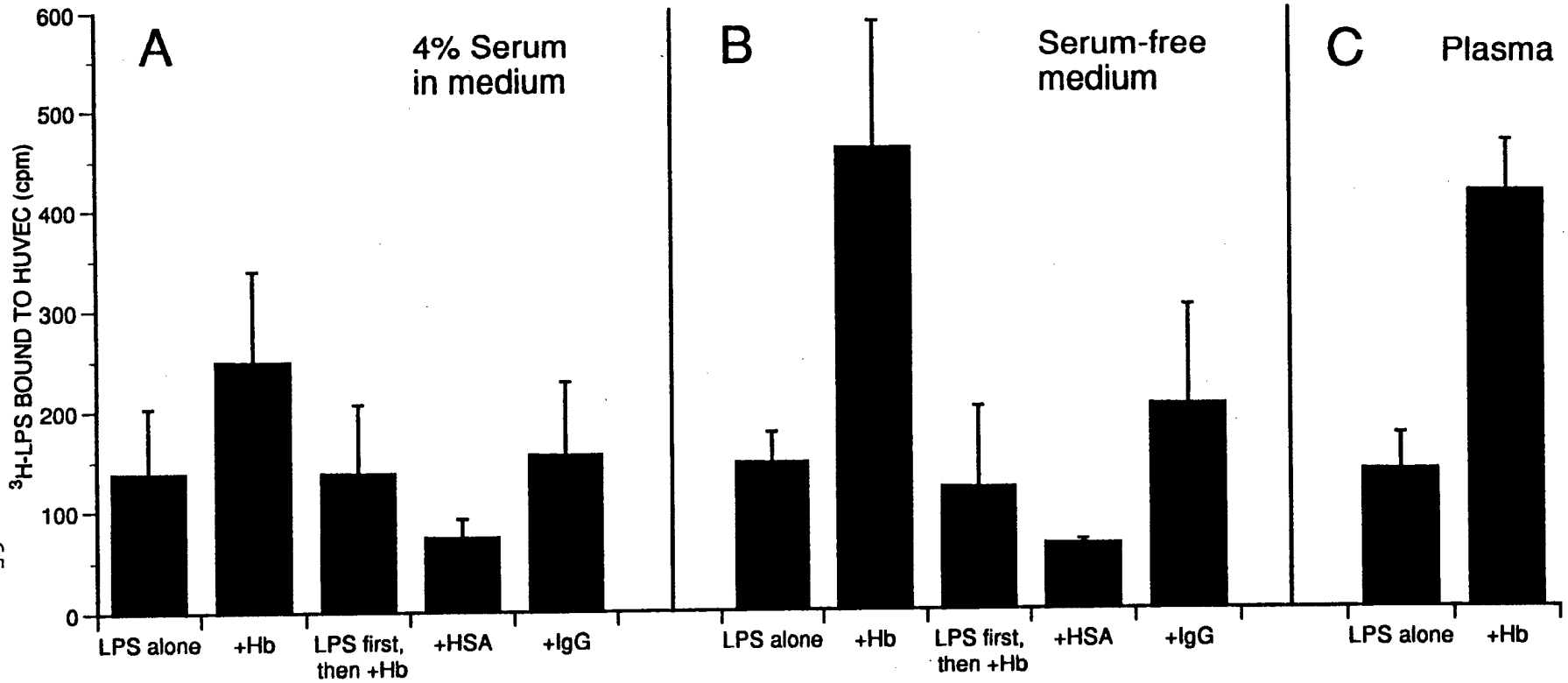
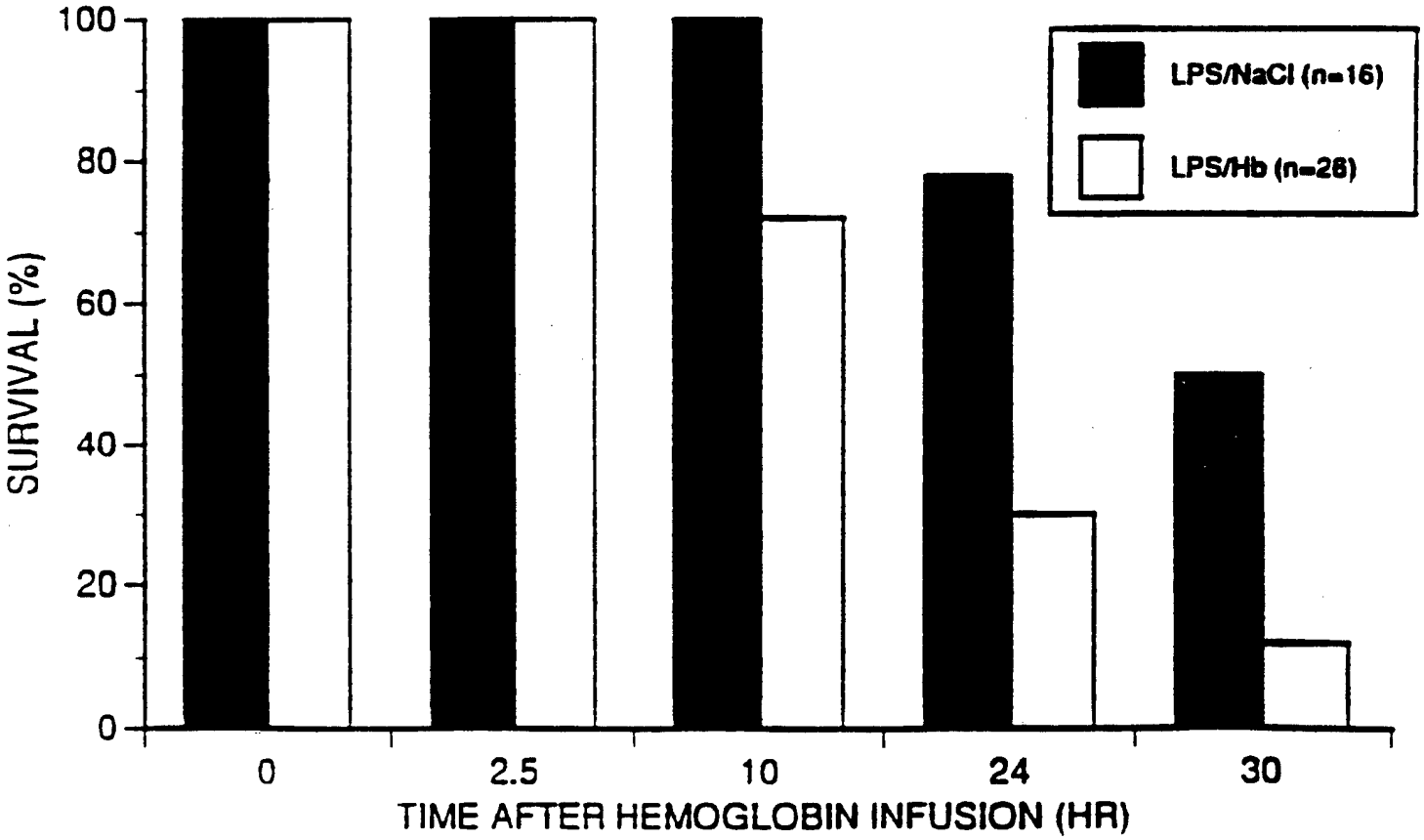
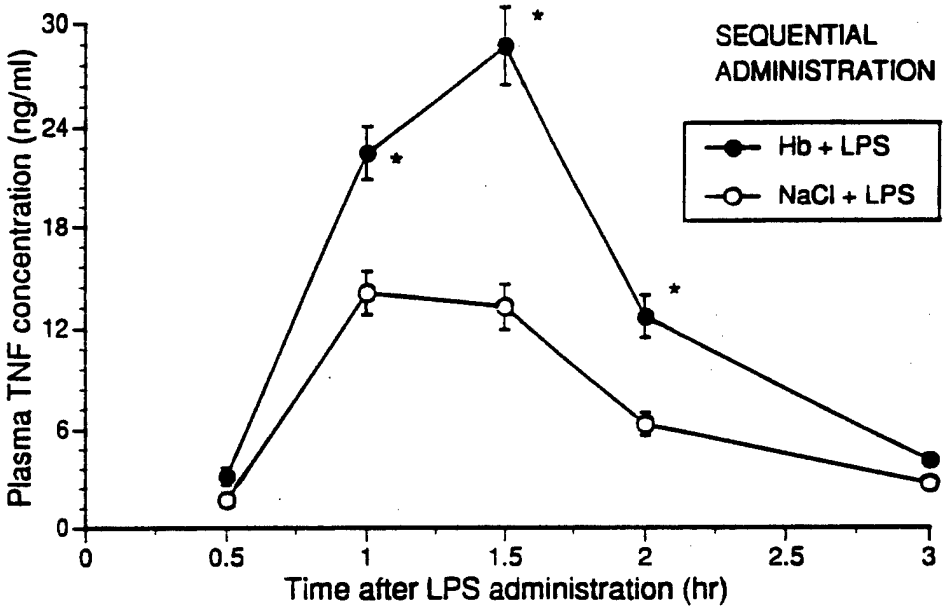

A
4% Serum in medium
B
Serum-free medium
C
Plasma
^{3}H-LPS BOUND TO HUVEC (cpm)
600
500
400
300
200
100
0
LPS alone
+Hb
LPS first, then +Hb
+HSA
+IgG
LPS alone
+Hb
LPS first, then +Hb
+HSA
+IgG
LPS alone
+Hb

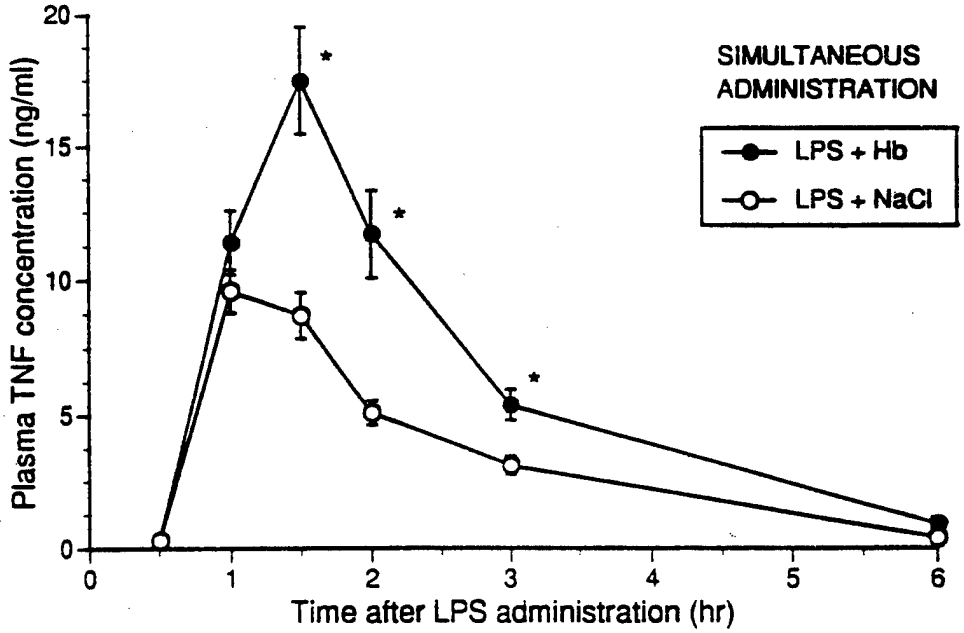
Fig. 18

LEVIN

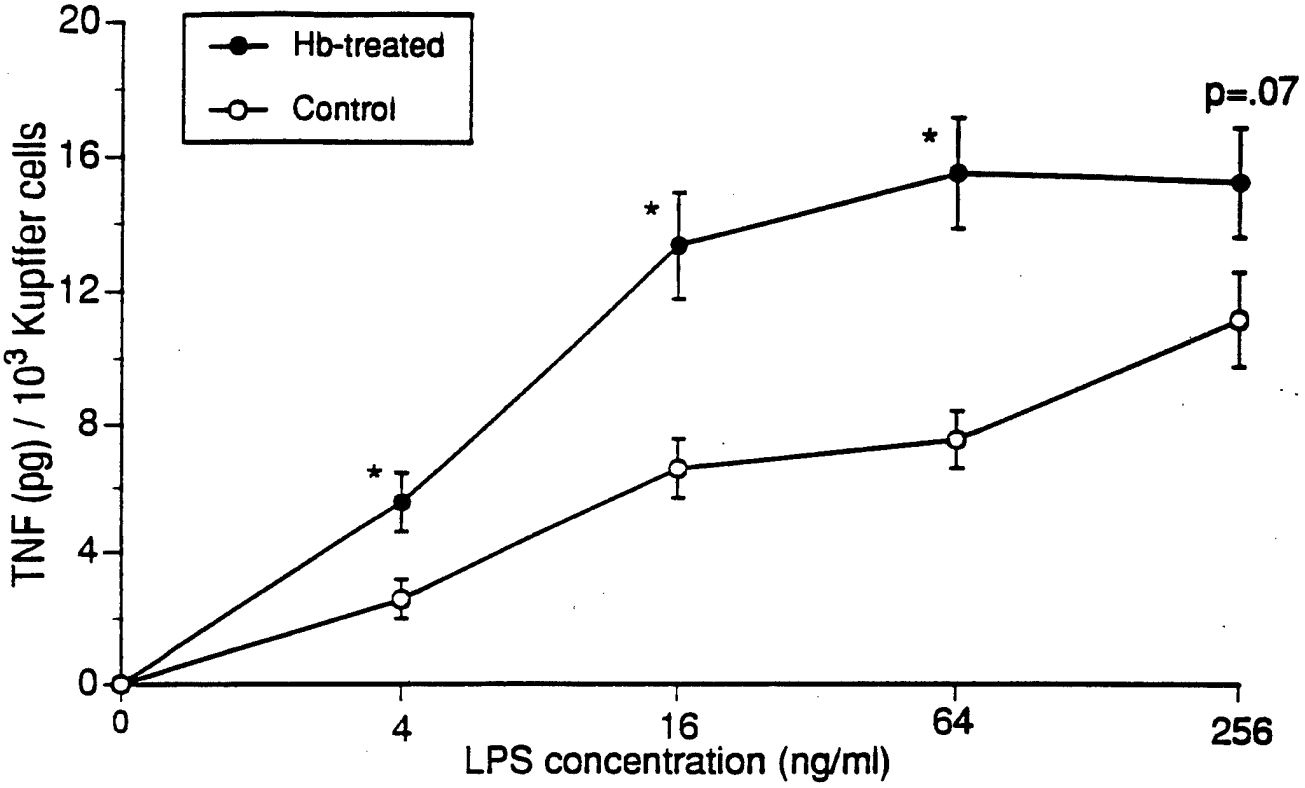

100
80
60
40
20
0
SURVIVAL (%)
LPS/NaCl (n=16)
LPS/Hb (n=28)
0
2.5
10
24
30
TIME AFTER HEMOGLOBIN INFUSION (HR)

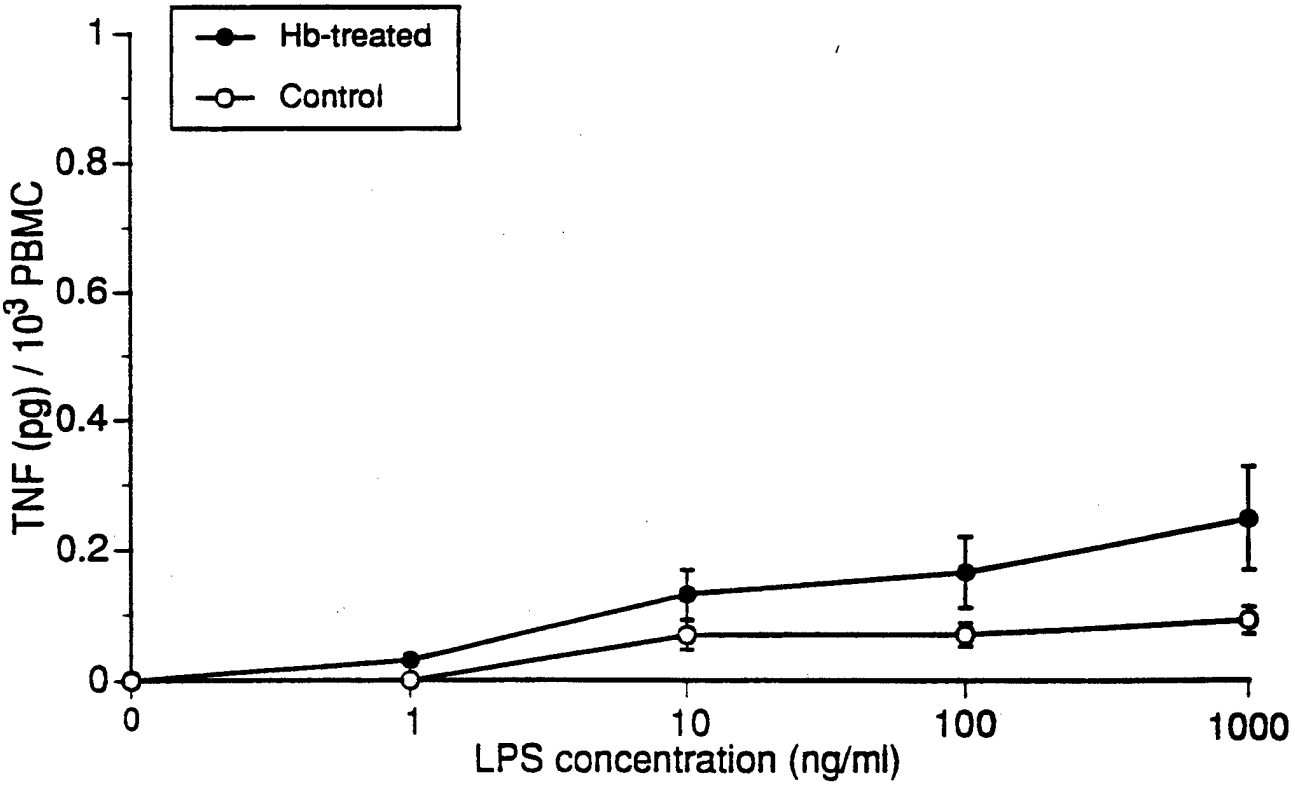
LEVIN
Fig. 19


SEQUENTIAL
ADMINISTRATION
Hb + LPS
NaCl + LPS
Plasma TNF concentration (ng/ml)
Time after LPS administration (hr)
0
6
12
18
24
30
0
0.5
1
1.5
2
2.5
3
*


SIMULTANEOUS
ADMINISTRATION
LPS + Hb
LPS + NaCl
Plasma TNF concentration (ng/ml)
Time after LPS administration (hr)
0
5
10
15
20
0
1
2
3
4
5
6
*

LEVIN
Fig. 20


Hb-treated
Control
p=.07
*
*
*
TNF (pg) / 10³ Kupffer cells
20
16
12
8
4
0
0
4
16
64
256
LPS concentration (ng/ml)


Hb-treated
Control
TNF (pg) / 10³ PBMC
1
0.8
0.6
0.4
0.2
0
0
1
10
100
1000
LPS concentration (ng/ml)

LEVIN
Fig. 21

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Roth RI, Su D, Child AH, Wainwright NR and Levin J. *Limulus* anti-lipopolysaccharide factor prevents mortality late in the course of endotoxemia. *J Infect Dis* 177:388-394, 1998.

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Levin J. The effects of bacterial endotoxins on human hemoglobin. Blood Substitutes & Related Products (IBC Symposium), September, 1994.

Levin J. Interaction of hemoglobin and bacterial endotoxins. Blood Substitutes & Related Products (IBC Symposium), October, 1995.

Su D, Roth RI, and Levin J. Hemoglobin enhances the mortality from bacterial endotoxin. Blood 1995; 86 (Supple 1): 445a. (Annual Meeting of the American Soc Hematology)

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Stowell C, Levin J, Spiess B, and Winslow R. Red blood cell substitutes. Annual Meeting, Amer Assoc of Blood Banks, October, 1997.

Personnel Who Received Pay from the Contract

Robert I. Roth, M.D., Ph.D., Co-Principal Investigator

Wieslaw Kaca, Ph.D., Post-Doctoral Research Fellow

Donghui Su, Ph.D.., Post-Doctoral Research Fellow

Georgiann Baker, B.S., Biological Laboratory Technologist

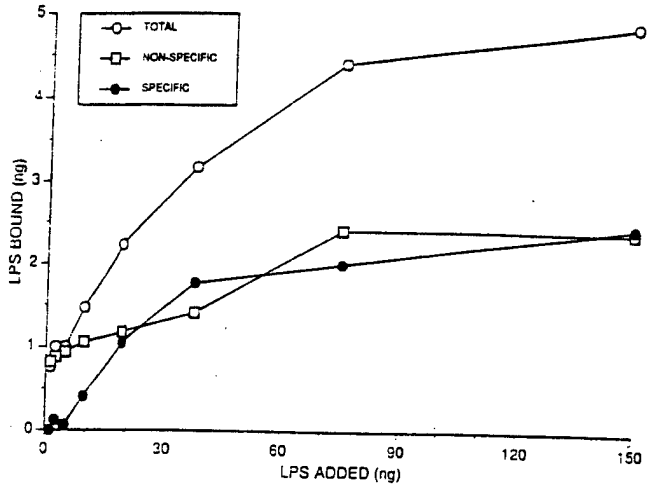

FIG. 1. **Binding of LPS to immobilized Hb.** $\alpha\alpha$Hb (1 μg/well) was immobilized in microtiter plate wells, and ^{125}I-LPS was added. Bound LPS was determined by gamma counting, and specific binding was calculated by subtracting bound ^{125}I-LPS in wells without Hb.

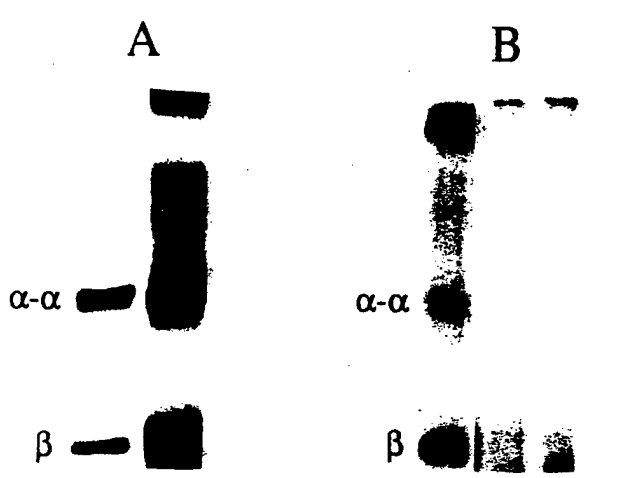

FIG. 2. **Photoaffinity labeling of Hb with ^{125}I-LPS-ASD.** ^{125}I-LPS-ASD was incubated with $\alpha\alpha$Hb, photolyzed with UV light, and electrophoresed in SDS and 2-mercaptoethanol. Following electrophoresis, the gel was stained with Coomassie Blue (*A, left lane*), dried, and subjected to autoradiography (*A, right lane*). Another photoaffinity-labeled $\alpha\alpha$Hb preparation from a separate experiment is shown (*B, left lane*), along with controls that consisted of an incubation mixture containing 100-fold excess unlabeled LPS as a blocking agent to demonstrate inhibition of specific binding (*B, middle lane*) and ^{125}I-LPS-ASD alone (*B, right lane*).

ing occurred with a calculated K_d of 4.7×10^{-4} g/liter (3.1×10^{-8} M, assuming a monomer molecular mass of 1.5×10^4 for *E. coli* LPS).

LPS Photoaffinity Labeling of Hb—In the presence of SDS, $\alpha\alpha$Hb electrophoresed as two bands, an-$\alpha\alpha$ cross-linked dimer of 33 kDa and monomer β chains of 16.5 kDa (Fig. 2*A, left lane*). Autoradiography of Coomassie Blue-stained gels reproducibly demonstrated binding of the photoaffinity LPS probe to both chains of $\alpha\alpha$Hb (Fig. 2*A, right lane* and 2*B, left lane*). Binding of the LPS photoaffinity probe to Hb was totally blocked by 100-fold excess unlabeled LPS (Fig. 2*B, middle lane*) indicating that the binding was specific. Although the LPS photoaffinity probe to Hb labeled both types of Hb chain, there was substantially more labeling of the β chains. When the monomer and dimer bands were excised from the gel and the associated counts/min were determined by gamma counting, 72% of counts/min were in the β band (range, 57–80%), and 28% were in the $\alpha\alpha$ dimer band (range, 20–43%) in three independent experiments. Binding of the LPS photoaffinity probe to native HbA_0 also was demonstrated (data not shown).

Ultrafiltration of Hb and LPS—87–89% of the *E. coli* LPS in Hb/LPS mixtures was filtered through a 300-kDa membrane (although in aqueous solutions, highly aggregated LPS typically has a molecular weight greater than 10^6), whereas only 10.2% of *E. coli* LPS alone was filterable (Table I). Similarly, 91–97% of the *P. mirabilis* LPS in Hb/LPS mixtures was filtered through the 300-kDa cut-off membrane, whereas only 15.6% of *P. mirabilis* LPS alone was filterable (Table I). This increase in LPS filterability was also demonstrated using radiolabeled LPS (^{125}I-labeled *P. mirabilis* LPS); only 22% of the LPS alone was filterable, but 78% of LPS in the presence of $\alpha\alpha$Hb was filterable (data not shown). 64–72% of the *P. mirabilis* S1959 LPS was filtered through a 100-kDa membrane in the presence of Hb, but LPS alone was not filterable (Table I). Approximately 90% of the total Hb protein in each of the three Hb/LPS mixtures, and from filtrates of Hb alone, was detected in filtrates of the 300- and 100-kDa membranes (data not shown).

Sucrose Centrifugation of LPS and Hb—High speed ultracentrifugation (52,000 × *g* for 4 h) of Hb alone demonstrated sedimentation of Hb part way into a 4–20% sucrose gradient. Whereas LPS alone sedimented to the bottom of the gradient, most of the LPS (80–95%) in Hb/LPS mixtures had a sedimentation rate similar to that of Hb (Fig. 3). In four independent experiments, Hb/LPS mixtures demonstrated co-migration of

TABLE I
Ultrafiltration of E. coli O26:B6 and P. mirabilis S1959 LPS, Hb, and Hb/LPS mixtures

Each experiment was performed three times and the mean ± S.D. is shown. Percent of LPS filtered was determined with the chromogenic LAL test. LPS was quantified with reference to standard curves consisting of the respective LPS/protein mixture prior to filtration (see "Experimental Procedures").

	E. coli LPS filtered (300-kDa[a] filter)	*P. mirabilis* LPS filtered	
		100-kDa[a] filter	300-kDa[a] filter
	%	%	
LPS alone	10.2 ± 2.3	0	15.6 ± 5.6
$\alpha\alpha$Hb alone	0[b]	0	0
$\alpha\alpha$Hb + LPS	87.3 ± 8.0	63.6 ± 18.7	97.1 ± 1.5
$\alpha\alpha$HbCO alone	0	0	0
$\alpha\alpha$HbCO + LPS	89.3 ± 1.5	71.1 ± 4.0	90.9 ± 4.5
HbA_0 alone	0	0	0
HbA_0 + LPS	88.1 ± 3.7	71.6 ± 8.8	93.5 ± 8.6

[a] Molecular mass cut-off of the filter.
[b] Lack of detectable LPS indicates that the starting preparations of Hb were endotoxin-free.

both components (Hb and LPS). Slow speed centrifugation (2,900 × *g* for 30 min) through 5% sucrose also demonstrated co-migration of Hb and LPS. 70.2% of LPS sedimented into the bottom fraction in the absence of protein, whereas only 4–11% sedimented in the presence of any of the three Hb preparations (Fig. 4). Conversely, only 10.7% of LPS alone remained above the sucrose layer, whereas, in the presence of Hb, 65–80% of LPS remained in the top layer. A similar redistribution of LPS from the bottom fraction (in the absence of protein) to the top fraction was observed in the presence of HSA. No detectable Hb or HSA entered the sucrose layer in either the absence or presence of LPS. Redistribution of LPS into the top layer was a saturable process, and binding of LPS to Hb occurred with a calculated K_d of 6.3×10^{-4} g/liter (6.3×10^{-8} M assuming a monomer molecular mass of approximately 10,000 Da for *S. typhimurium* Rc LPS) (data not shown). This number is in close agreement with the K_d calculated from the microtiter plate binding assay (4.7×10^{-4} g/liter) described above.

Nondenaturing Gel Electrophoresis of LPS and Hb—100% of ^{14}C-labeled *S. typhimurium* LPS alone remained within the first 9 mm of the gel (gel pieces 1–3), whereas 43% of the LPS

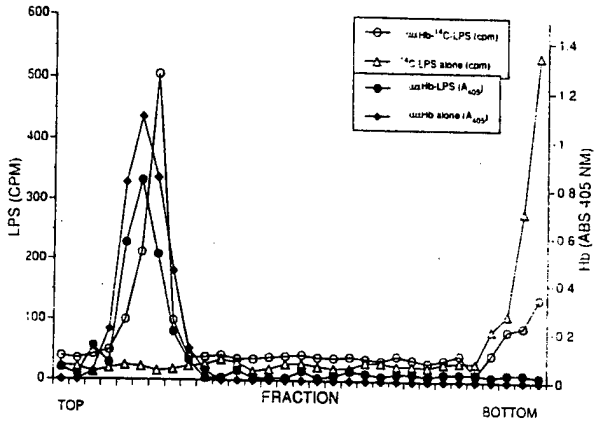

FIG. 3. **Sucrose density centrifugation of Hb/LPS.** ^{14}C-LPS was incubated with $\alpha\alpha$Hb (100 mg/ml), and the mixture was centrifuged through a 4–20% continuous sucrose gradient. 0.4-ml fractions were assayed for hemoglobin by absorbance at 405 nm (*closed symbols*) and for LPS by scintillation counting (*open symbols*).

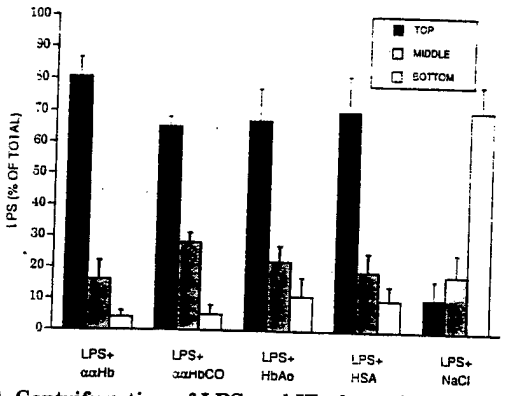

FIG. 4. **Centrifugation of LPS and Hb through 5% sucrose.** ^{14}C-Labeled LPS was incubated with $\alpha\alpha$Hb, $\alpha\alpha$HbCO, HbA_0, or HSA. These LPS/protein mixtures, and LPS in NaCl, were centrifuged through a layer of 5% sucrose as described under "Materials and Methods." ^{14}C-Labeled LPS was measured by scintillation counting in the top (*solid columns*), middle (*stippled columns*), and bottom (*open columns*) zones of the centrifuged samples. Results are expressed as the mean ± S.D. of four independent experiments.

in the $\alpha\alpha$Hb/LPS mixture migrated farther into the gel and co-migrated with $\alpha\alpha$Hb (gel pieces 6–17) (Fig. 5). Recovery of the total applied counts/min from all the gel pieces was 93%. A second electrophoresis experiment with ^{14}C-labeled *S. typhimurium* LPS demonstrated that 45% of the LPS co-migrated with the $\alpha\alpha$Hb (data not shown), and an identical experiment performed with ^{125}I-labeled *E. coli* LPS demonstrated 23% co-migration (data not shown).

Precipitation of LPS and Hb by Ethanol—At each of the temperatures studied (4, 20, or 37 °C) and both in the presence and absence of Hb, 90–100% of *E. coli* O26 LPS or *P. mirabilis* S1959 LPS was precipitated by ethanol (data not shown). Precipitation of Hb alone was variable (13.9–37.5%). However, in almost all conditions studied, the presence of LPS increased the amount of precipitated Hb protein (Table II). Mixtures of *E. coli* LPS with each of the Hb solutions, incubated and precipitated at each of the three temperatures, demonstrated from 14.1 to 42.5% more Hb precipitated than when Hb was precipitated in the absence of LPS. Similarly, mixtures of *P. mirabilis* S1959 LPS and each of the Hb preparations, incubated and precipitated at 20 and 37 °C, demonstrated increased Hb precipitation (from 6.7 to 12.6% more protein than Hb alone) (Table II). At 4 °C, the HbA_0/*P. mirabilis* LPS mixture demonstrated increased precipitation of HbA_0 (22.5%) compared with HbA_0 precipitation in the absence of LPS. In contrast, the precipitation

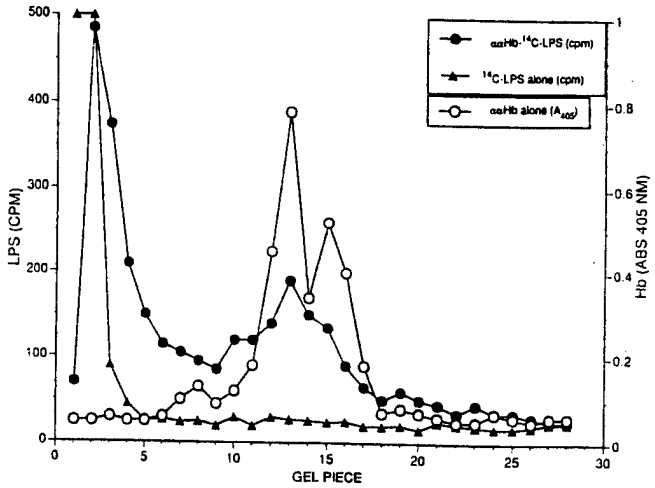

FIG. 5. **Electrophoresis of LPS and Hb.** ^{14}C-Labeled LPS was incubated with $\alpha\alpha$Hb, and the $\alpha\alpha$Hb/LPS mixture or LPS alone was electrophoresed in polyacrylamide in the absence of SDS, as described under "Materials and Methods." ^{14}C-Labeled LPS was measured by scintillation counting of gel pieces (*closed symbols*), and $\alpha\alpha$Hb was monitored by absorbance at 405 nm (*open circles*).

TABLE II

Percent of cell-free hemoglobin precipitated by ethanol in the absence and presence of P. mirabilis S1959 or E. coli O26:B6 endotoxins (LPS)

The mean value of eight replicate precipitations ± S.D. is shown. Percent of Hb precipitated was determined by measurement of protein.

Type of Hb	Temperature[a]	Hb alone	Hb + LPS (*P. mirabilis*)	Hb + LPS (*E. coli* O26)
	°C[b]			
$\alpha\alpha$Hb	4	37.5 ± 2.6	38.3 ± 0.8 (0.8)[b]	67.3 ± 1.9 (29.8)
$\alpha\alpha$Hb	20	26.8 ± 5.9	35.9 ± 10.8 (9.1)	69.3 ± 2.5 (42.5)
$\alpha\alpha$Hb	37	22.7 ± 8.7	32.1 ± 4.9 (9.4)	56.9 ± 6.5 (34.2)
$\alpha\alpha$HbCO	4	34.1 ± 1.9	35.4 ± 0.8 (1.3)	69.7 ± 1.9 (35.6)
$\alpha\alpha$HbCO	20	25.4 ± 5.8	33.3 ± 7.9 (7.9)	66.8 ± 17.2 (41.4)
$\alpha\alpha$HbCO	37	26.2 ± 6.9	32.9 ± 5.6 (6.7)	59.5 ± 6.5 (33.3)
HbA_0	4	17.5 ± 0.6	40.0 ± 1.0 (22.5)	31.6 ± 0.6 (14.1)
HbA_0	20	13.9 ± 5.8	26.5 ± 13.5 (12.6)	45.9 ± 6.2 (32.0)
HbA_0	37	20.3 ± 10.1	31.9 ± 1.9 (11.6)	44.5 ± 4.4 (24.2)

[a] Temperature of incubation and precipitation.

[b] Numbers in parentheses indicate increase in percent of Hb precipitated by ethanol (67%, final concentration) in the presence of endotoxin.

of $\alpha\alpha$Hb or $\alpha\alpha$HbCO was not significantly altered by *P. mirabilis* LPS at 4 °C. *E. coli* O26:B6 LPS co-precipitated significantly more of each Hb than *P. mirabilis* LPS at all conditions tested, except for the incubation of HbA_0 with LPS at 4 °C (Table II).

LAL Reactivity of Hb and LPS Mixtures—Using the chromogenic LAL test with *Limulus* lysate, which had been diluted 20-fold in order to expand the measurable range of LPS concentrations, *E. coli* O26:B6 LPS was assayed alone and in the presence of Hb or HSA (Fig. 6*A*). All three Hb preparations (and HSA) resulted in enhanced activation of LAL over a wide range of LPS concentrations. Sensitivity of LAL for low concentrations of LPS was increased approximately 10-fold in the presence of proteins. Comparable enhancement of the biological activity of LPS in the LAL test by each of the three Hb preparations and by HSA also was demonstrated using *P. mirabilis* S1959 LPS (Fig. 6*B*). Similar enhancement effects were ob-

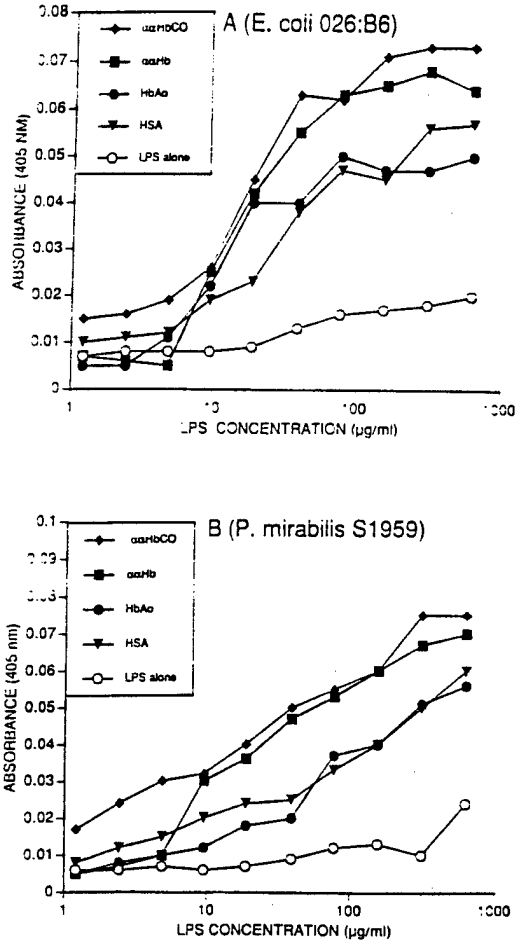

FIG. 6. **Enhancement of LPS activation of *Limulus* amebocyte lysate by Hb.** Dilutions of *E. coli* O26:B6 LPS (*A*) or *P. mirabilis* S1959 LPS (*B*) in ααHbCO (◆), ααHb (■), HbA_o (●), HSA (▼) or NaCl (○), were assayed with the chromogenic LAL test. Absorbances at 405 nm were measured at 5 min. All protein concentrations were 1 mg ml. Incubations were performed in triplicate, and the mean is shown.

tained from three independent experiments. The enhancement by each Hb of LAL activation also was demonstrated with the gelation LAL test, using undiluted *Limulus* lysate for maximum LPS sensitivity, and enhancement was shown to be dependent on protein concentration (Fig. 7). Prominent enhancement (2–10-fold) was observed over a log range of concentrations for each Hb (from 0.2 to 2 mg/ml Hb). At 100 μg/ml Hb, less than 2-fold enhancement was demonstrated with ααHb and HbA_o; ααHbCO at 100 μg/ml did not demonstrate enhancement. Three independent experiments failed to demonstrate enhancement by any tested concentration of HSA (from 0.01 to 2 mg/ml) in the gelation LAL test: this is in contrast to the reproducible enhancement by HSA demonstrated utilizing diluted LAL in the chromogenic test.

DISCUSSION

ααHb is a cell-free preparation of a derivatized human hemoglobin (cross-linked between α chains), which has adequate oxygen carrying and releasing properties and also an acceptable *in vivo* half-life (4–24 h in various animals) (1, 26–29). Despite these favorable characteristics, *in vivo* animal studies with ααHb and other purified cross-linked hemoglobin preparations have demonstrated significant toxicity (6, 7, 30, 31), thus limiting their potential clinical use. However, it is uncertain whether ααHb is intrinsically toxic, or if the previously described *in vivo* toxicity has primarily resulted from associated endotoxin or contaminating stromal phospholipids (6).

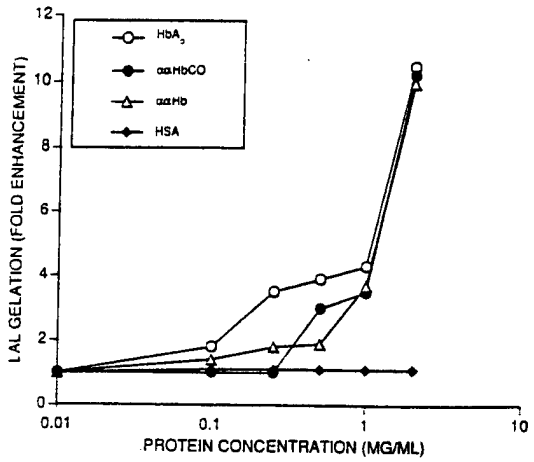

FIG. 7. **Enhancement of LPS activation of *Limulus* amebocyte lysate by Hb.** Mixtures of LPS (100 pg/ml *E. coli* O26:B6) and HbA_o (○), ααHbCO (●), ααHb (△), or HSA (◆) were assayed with LAL, using gelation as the end point. Protein concentrations ranged from 0.01 to 2 mg/ml. Enhancement of activation of LAL was calculated by comparison of the gelation time of each mixture with the gelation times for LPS solutions in 0.9% NaCl. 100 pg/ml LPS in 0.9% NaCl solution gelled *Limulus* amebocyte lysate in 2.5 h. Similar results were obtained from each of three independent experiments.

Toxicity due to associated LPS has been described previously (6, 9, 10), and evidence for synergistic toxicity of LPS and Hb also has been reported (10, 32). Due to the large volumes of Hb that would be infused into a patient during resuscitation, LPS contamination of Hb would be a potentially major limitation to the clinical use of solutions of Hb.

Preliminary experiments in our laboratory suggested that formation of ααHb·LPS complexes could contribute to the observed *in vivo* toxicity of preparations of Hb by modification of the physical structure and biological activity of contaminating LPS. Therefore, we performed experiments to determine if physical interactions between Hb and LPS were demonstrable and if such interactions altered the biological activity of LPS. Six different techniques provided evidence to support the conclusion that Hb and LPS formed stable complexes. Firstly, LPS bound to immobilized Hb in a saturable manner. The calculated K_d (4.7×10^{-4} g/liter based on the microtiter plate binding assay, and 6.3×10^{-4} g/liter based on the sucrose centrifugation assay) indicated that the interaction is of moderate affinity. When expressed as molar values, these binding constants of LPS for Hb were in the same range as binding constants reported for other LPS binding proteins (33–35). Second, specific binding of the LPS photoaffinity probe ^{125}I-LPS-ASD to Hb confirmed complex formation, and demonstrated that binding of LPS to the β chains of Hb is particularly prominent. Third, a portion of the LPS in the presence of Hb demonstrated increased electrophoretic mobility, and co-electrophoresed with Hb. This result is consistent with a process of LPS disaggregation and Hb·LPS complex formation. Fourth, in the presence of Hb (ααHb, ααHbCO, or HbA_o) the majorities (approximately 70–90%) of both LPSs utilized (*E. coli* O26:B6 or *P. mirabilis* S1959) were detectable in filtrates of 300- and 100-kDa ultrafiltration membranes, in contrast to the lack of filterability of LPS in the absence of Hb. The filterability of LPS in the presence of Hb was consistent with the presence of relatively low molecular weight Hb·LPS complexes. This is in contrast to the high molecular weight aggregation state of LPS alone (typically $>10^6$). Fifth, the reduction in sedimentation velocity, determined by sucrose centrifugation of each LPS after incubation with each of the three Hb preparations, further supported the conclusion that Hb altered the physical properties of LPS, and the comigration of LPS and Hb provided additional evidence that Hb

and LPS formed stable complexes. Finally, the co-precipitation by ethanol of each of the three Hb preparations with each LPS provided evidence that LPS altered the physical properties of Hb and also was consistent with the presence of stable Hb·LPS complexes. Hb/LPS ratios differed considerably for performance of experiments using these models because of the variable methods required for Hb and LPS detection, yet each of these models supported the conclusion that complex formation had occurred.

Formation of Hb·LPS complexes was demonstrated for native HbA_o as well as for the derivatized cross-linked $\alpha\alpha$Hb. This finding provided further evidence for the general conclusion that $\alpha\alpha$Hb is similar physiologically to native hemoglobin (29). Additionally, this observation suggests that native hemoglobin, released into the circulation by *in vivo* hemolysis of erythrocytes (*e.g.* as can be observed during Gram-negative bacterial sepsis), may interact with circulating endotoxins.

Interaction of LPS with cross-linked and native Hb preparations also was associated with increased biological activity (enhanced LAL activation) of LPS. In the presence of all three Hb preparations, LPS activated LAL both more rapidly and at lower concentrations than in the absence of Hb. These results are in agreement with the previous preliminary finding (36) that preparations of $\alpha\alpha$Hb (ranging from 0.001–100 mg/ml) enhanced the ability of *E. coli* lipopolysaccharide (O55:B5) to activate LAL. Confirmation of increased biological activity of LPS, in the presence of Hb, also has been provided by the observations that generation of mononuclear cell tissue factor by LPS is enhanced by Hb (37) and that generation of tissue factor from endothelial cells is enhanced by Hb (38). Lipid A was capable of interacting with Hb to produce an increase in LPS biological activity, although the -fold enhancement of lipid A activity by Hb was less than that observed with complete LPS.[1] Hb·LPS complex formation and LAL enhancement by $\alpha\alpha$HbCO, results which were similar to those observed with $\alpha\alpha$Hb and HbA_o, established that these properties of Hb do not involve methemoglobin production and are not related to the state of oxygenation of Hb.

In contrast to the reproducible enhancement effect of Hb, our finding that enhancement of LPS biological activity by HSA was observed with one of our assay conditions (the diluted LAL chromogenic assay) but not with another (the undiluted gelation assay) suggests that the Hb-LPS and Hb-HSA interactions may not be similar. Variable effects of HSA on LPS biological activity have been described; the biological activity of LPS in the presence of HSA previously has been shown to be increased (39), decreased (40), or both increased and decreased, depending on HSA concentration (41).

The increased reactivity of LPS, when complexed with Hb, the associated decrease in molecular weight and density of LPS, and the altered electrophoretic mobility of LPS may be explained by a detergent-like effect of Hb on LPS; *i.e.* Hb may reduce the size of the LPS aggregates, making LPS more soluble and more biologically available for activation of the LAL enzymatic cascade. Disaggregation of LPS is a well recognized phenomenon in plasma (42, 43), although this process previously has been demonstrated to result in detoxification of LPS (43). In contrast to the effect of Hb, other proteins that bind LPS and result in altered LPS biological activity, such as melittin (44), bacterial outer membrane 39-kDa protein (45), lysozyme (33), complement proteins (46), bactericidal/permeability-increasing protein (47), or polymyxin B (48), cause a decrease in LPS toxicity. Although the mechanism of Hb enhancement of LPS biological activity is likely to involve LPS disaggregation, it is also possible that LPS undergoes a chemical modification in the presence of Hb, perhaps similar to the process of phospholipid peroxidation that has been demonstrated when oxyhemoglobin binds phospholipid (49).

The increased biological activity (*e.g.* in LAL activation) of LPS in the presence of $\alpha\alpha$Hb and the other Hb preparations is of potential physiological significance since $\alpha\alpha$Hb would likely be infused into trauma patients with concomitant endotoxemia. In rabbits, LPS and $\alpha\alpha$Hb have been shown to have synergistic toxicity (10), and, in dogs, impure hemoglobin, produced by hemolysis of red blood cells, was shown to enhance the toxicity of infused LPS (32). Importantly, the deleterious effects of interactions of LPS with blood cells (*e.g.* activation of mononuclear cells) and the endothelium (*e.g.* induction of a procoagulant state) might be augmented in the presence of $\alpha\alpha$Hb. A patient requiring $\alpha\alpha$Hb for resuscitation would potentially require a plasma concentration of 50 mg/ml (5 g/dl) of $\alpha\alpha$Hb in order to provide adequate oxygen carrying capacity. This is a concentration 25-fold greater than that demonstrated in Fig. 7 to enhance LAL gelation 10-fold. Therefore, enhancement of LPS biological activity by $\alpha\alpha$Hb would constitute a serious clinical risk if the patient was endotoxemic. Accordingly, successful development and clinical utilization of $\alpha\alpha$Hb as a red blood cell substitute will depend on a more complete understanding of the interaction between $\alpha\alpha$Hb and LPS.

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RESEARCH

Human hemoglobin increases the biological activity of bacterial lipopolysaccharides in activation of *Limulus* amebocyte lysate and stimulation of tissue factor production by endothelial cells in vitro

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SUMMARY. **Previous studies have demonstrated that hemoglobin (Hb) and bacterial endotoxin (lipopolysaccharide, LPS) form stable complexes and result in disaggregation of macromolecular LPS. To examine the effect of complex formation on LPS biological activity, we investigated the ability of Hb to alter LPS-induced activation of the coagulation cascade of *Limulus* amebocyte lysate (LAL) and expression of tissue factor from human endothelial cells. Both native HbA_0 and derivatized (covalently cross-linked) hemoglobin resulted in prominent enhancement of LAL activation and endothelial cell tissue factor production by *Proteus mirabilis* LPS. No substantial differences were observed between the enhancement effect of Hb on *P. mirabilis* smooth and rough LPS, indicating a dominant role for the lipid A component of LPS. Rough (Re) *Salmonella minnesota* 595 LPS also demonstrated both enhanced activation of LAL and stimulation of endothelial cell tissue factor in the presence of Hb. In contrast, neither lipid A nor singly dephosphorylated or partially deacylated Re LPS manifested significant enhancement of LAL activation by Hb, and partially deacylated Re LPS showed no enhancement of endothelial cell tissue factor by Hb. These results suggest that the Kdo moieties, as well as the phosphate residues and fatty acyl moieties of lipid A, may be involved in the interaction of Hb with LPS. Comparison of Hb with other endotoxin binding proteins for ability to cause enhancement of LPS biological activity demonstrated more prominent enhancement with lipopolysaccharide binding protein (LBP) than that observed with Hb, lesser enhancement with albumin, and no enhancement effect with IgG or transferrin.**

The ability of hemoglobin (Hb) solutions, in the absence of erythrocytes, to act as oxygen carriers has been recognized for several decades.[1] Recently, highly purified human Hb preparations have been produced that demonstrate adequate oxygen binding and releasing properties,[2–4] and are being developed for potential use as an oxygen-transporting resuscitation fluid.[5,6] These materials have been rigorously purified of red cell stromal (lipid) contaminants, and in some instances have been covalently cross-linked between chains of the Hb tetramer to maintain the tetrameric structure outside of the erythrocyte and prolong intravascular persistence.[7] However, Hb, removed from the erythrocytic milieu, has been associated with many toxic effects when administered in vivo, prominent among which are fever, hypertension, renal failure, hepatic necrosis and coagulopathy.[8–11] Several potential causes of these toxicities have been described, including the association of multisystem dysfunction with contamination of Hb by residual erythrocyte phospholipids and/or bacterial endotoxin lipopolysaccharide (LPS).[8,12–14]

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Recently, we have shown that complex formation occurs between Hb and LPS, resulting in a significant decrease in the aggregate molecular weight of LPS, and postulated that Hb-LPS complex formation was partially responsible for the observed toxicities of in vivo infusions of Hb.[15] In addition, in preliminary experiments the biological activity of LPS was increased, as demonstrated by enhanced LPS activation of *Limulus* amebocyte lysate (LAL) in the presence of Hb.[15] The formation of Hb-LPS complexes also resulted in enhanced LPS activation of human peripheral blood mononuclear cells.[16] We have hypothesized that the binding and disaggregation of LPS by Hb increases LPS solubility, resulting in augmented biological activity. However, the mechanism and molecular constituents of LPS required for this effect have not been identified. The present study was designed to evaluate the requirements for different components of LPS in producing the enhancement effect of Hb.

Proteus mirabilis LPS, differing in polysaccharide composition, were studied because they provided a series of probes for evaluation of the role of the O chain polysaccharides in the enhancement process. Rough (Re) *Salmonella minnesota* 595 LPS and selected chemical derivatives were used to evaluate the contribution of fine structural components of the core and lipid A moieties. Hb preparations studied have included cross-linked human Hb (ααHb) because this Hb preparation is being developed as a red blood cell substitute, and native, unmodified HbA_o to ensure that we were studying an intrinsic property of Hb. We have also investigated carbonmonoxyhemoglobin (ααHbCO) as a form of Hb that would have a greatly reduced propensity to generate methemoglobin. Our data have demonstrated that the hydrophobic components of LPS, i.e. lipid A and the deep rough portion of the core region of LPS, are responsible for the interactions with human Hb that lead to the enhancement of LPS activity in the LAL assay and stimulation of the production of tissue factor by endothelial cells.

MATERIALS AND METHODS

Reagents

Sterile, endotoxin-free water and 0.9% NaCl were purchased from Travenol Laboratories (Deerfield, IL, USA). RNAse and DNAse were purchased from Sigma Chemical Co. (St Louis, MO, USA). Human serum albumin (HSA) (25%, for injection) was purchased from Nybcen (New York, NY, USA), human immunoglobulin (IgG) (185 mg/ml) from Armour Pharmaceutical Co. (Kankakee, IL, USA) and human transferrin from Calbiochem (La Jolla, CA, USA).

Glassware

All glassware was rendered endotoxin-free by heating at 190°C in a dry oven for 4 h.

Hemoglobin

Human Hb was prepared and purified, as described previously,[2] by collaborators at the Blood Research Division of the Letterman Army Institute of Research (BRD/LAIR), San Francisco, CA, USA. Human Hb was covalently cross-linked between α chains with bis(3,5-dibromosalicyl)fumarate (ααHb). The ααHb stock solution was 9.6 g/dl, pH 7.4 in Ringers acetate, and contained less than 0.4 EU/ml endotoxin (referenced to *Escherichia coli* lipopolysaccharide B, O55:B5, Difco Laboratories, Detroit, MI, USA), as determined by LAL.[17] The ααHb stock solution was stored at –70°C, and diluted with sterile, pyrogen-free 0.9% NaCl prior to use. ααHbCO (95% HbCO), produced by incubation of the ααHb solution with CO, also was at 9.6 g/dl, pH 7.4. Purified non-crosslinked A_o (HbA_o), 8.4 g/dl, was prepared from Hb by ion exchange HPLC, as described previously.[3]

Lipopolysaccharide binding protein

Purified rabbit LPS binding protein (LBP) was the generous gift of Dr Peter Tobias, The Scripps Research Institute, La Jolla, CA, USA.

Bacterial LPS

Proteus LPS

P. mirabilis S 1959 smooth LPS, Ra type (R110) and Re type (R45) rough mutants of S1959, and *P. mirabilis* O3 smooth LPS were prepared at the Institute of Microbiology and Immunology, University of Lodz, Poland. *P. mirabilis* R45 LPS contains only lipid A, two residues of 2-keto-3-deoxyoctulosonic acid (Kdo) and two 4-amino-arabinose (Ara4N) residues.[18,19] *P. mirabilis* R110 LPS contains the complete core oligosaccharide linked to lipid A.[20] Smooth LPS were extracted with phenol-water according to the Westphal method,[21] whereas rough LPS were extracted by the phenol-chloroform-petroleum ether method (PCP) according to Galanos.[22] Crude *Proteus* LPS were further purified by sequential treatment with RNAse and DNAse, followed by ultracentrifugation at 100,000 x g for 3 h, as described previously.[23]

Salmonella LPS

The deep rough *S. minnesota* R 595 LPS was extracted by the PCP method[22] and then, in order to study the role of the hydrophobic part of endotoxin (lipid A) on LAL activation, a series of partially chemically degraded derivatives were prepared as follows (see Table for LPS compositions).

Lipid A

S. minnesota R 595 lipid A was prepared from 134 mg of R 595 LPS by hydrolysis of the Kdo and Ara4N

residues with sodium acetate (pH 4.4) for 1 h at 100°C.[24] The hydrolysate was dialysed to obtain purified lipid A (67% yield) and then lyophilized and stored at 4°C.

Monophosphoryl lipid A

Monophosphoryl lipid A (MPL) was obtained from 200 mg of R 595 LPS by hydrolysis with 0.1 N HCl for 45 min at 100°C, conditions known to remove the phosphate residue from the reducing glucosamine,[25] and the Kdo and Ara4N residues from non-reducing glucosamine. The MPL hydrolysate was centrifuged at 10,000 x g for 30 min, washed twice with water, and then lyophilized (58% yield).

Singly deacylated LPS (OH37 LPS)

OH37 LPS was produced from 380 mg of R 595 LPS by hydrolysis of a single ester-bound 3-hydroxy-tetradecanoyl fatty acid from the reducing glucosamine of LPS with 0.2 N NaOH for 30 min at 37°C; 93% removal of this fatty acid has been demonstrated with this procedure.[25] The hydrolysate was then cooled to 4°C and neutralized to pH 6.5 with 0.1 N HCl. Released fatty acids were extracted with $CHCl_3$/MeOH (2:1) followed by precipitation of OH37 LPS with EtOH/acetone (2:1) at 0°C. The OH37 LPS sediment was centrifuged at 10,000 x g for 30 min, washed twice with cold EtOH, resuspended in water, and lyophilized (61% yield).

Multiply deacylated LPS (OH56 LPS)

OH56 LPS was produced from 190 mg of OH37 LPS by hydrolysis in 0.2 N NaOH for 60 min at 56°C.[25] The hydrolysate was cooled to 4°C, and then neutralized to pH 6.5 with 0.1 N HCl, precipitated by EtOH/acetone (2:1) at 4°C, and centrifuged at 10,000 x g for 30 min. The precipitate was washed twice with cold EtOH, resuspended in water and lyophilized (50% yield).

Salmonella abortus equi

S. abortus equi LPS was the generous gift of Dr Chris Galanos, Max-Planck Institute, Freiburg, Germany.

E. coli LPS

E. coli O26:B6 (Westphal preparation obtained by hot phenol-water extraction[21] was purchased from Difco Laboratories (Detroit, MI, USA). This crude *E. coli* LPS was further purified as described above for *Proteus* LPS.[23] For some experiments, purified *E. coli* O26 LPS then was electrodialysed at 0.47 mA for 8 h at 4°C and aliquots were neutralized with triethylamine, 0.01 N NaOH or 0.1 M $CaCl_2$ to form the triethylamine, sodium and calcium salts,[26] respectively. *E. coli* F515 LPS, prepared from the rough strain *E. coli* F515 (Re), was the generous gift of Dr Chris Galanos, Max-Planck Institute, Freiburg, Germany.

Rhodobacter LPS

Non-enterobacterial *Rhodobacter spheroides* ATCC 17023, *Rhodobacter capsulatus* 37b4 and *Rhodopseudomonas viridis* LPS were kindly provided by Dr Hubert Mayer, Max-Planck Institute, Freiburg, Germany.

Chemical analysis

Colorimetric methods were used to determine the con-

Table. Chemical composition of *S. minnesota* 595 LPS and its chemically degraded derivatives

Components (nmol/mg LPS)*		LPS				
		595	OH37	OH56	MPL	Lipid A
Kdo		655	825	811	6	53
$PO(OH)_2$		1160	1733	749	897	1052
C_{12}	Total	401†	598	76†	1137	530
	Ester	439	472	87	500	447
C_{14}	Total	214	147	0†	471	529
	Ester	76	100	14	119	167
C_{14}-OH	Total	1415	1278	703	2146	1623
	Ester	650	22	0	1092	812
C_{16}	Total	205	163	54†	214	154†
	Ester	155	155	63	117	182

*2-keto-3-deoxy-octulosonic acid (Kdo) and phosphate ($PO(OH)_2$) residues were determined in hydrolysates of LPS utilizing colorimetric reactions with thiobarbituric acid and molybdate complexes, respectively; fatty acids (C_{12}, dodecanoic acid; C_{14}, tetradecanoic acid, C_{14}-OH, 3-hydroxy-tetradecanoic acid, and C_{16}, hexadecanoic acid) were determined as their methyl esters, in basic (ester bound) or acid (total = ester and amide bound) hydrolysates of LPS.

†Determination of total fatty acids is slightly underestimated due to chemical degradation during hydrolysis.

Abbreviations: 595, untreated *S. minnesota* 595 LPS (Mr 2867‡); OH37, singly deacylated 595 LPS (Mr 2641); OH56, multiply deacylated 595 LPS (Mr 1785); MPL, monophosphoryl lipid A (partially dephosphorylated 595 LPS) (Mr 2094); lipid A, product after acetic acid hydrolysis of LPS 595 (Mr 2298).

‡Monomer molecular mass (Mr) of each LPS was estimated, assuming complete substitution of each structural component.

tent of Kdo[24] and phosphate residues;[27] gas-liquid chromatography (GLC) was performed for analysis of methyl esters of fatty acids.[28]

Turbidity measurements

0.18 ml of LPS solutions (1 mg/ml in 0.9% NaCl), in the absence or presence of ααHb (0.01–1 mg/ml) was incubated for 90 min at 37°C, and absorbance at 620 nm was determined, as a measurement of turbidity, in a temperature-controlled plate reader (Kinetic-QCL, Whittaker Bioproducts Inc., Walkersville, MD, USA).

Limulus amebocyte lysate

Amebocyte lysates were prepared from *Limulus polyphemus* (the North American horseshoe crab) by lysis of washed amebocytes in distilled water, as described previously.[17,29] Limuli were obtained from the Department of Marine Resources, Marine Biological Laboratory, Woods Hole, MA, USA.

Chromogenic substrate

Chromogenic substrate S-2423 (AB Kabi Vitrum,

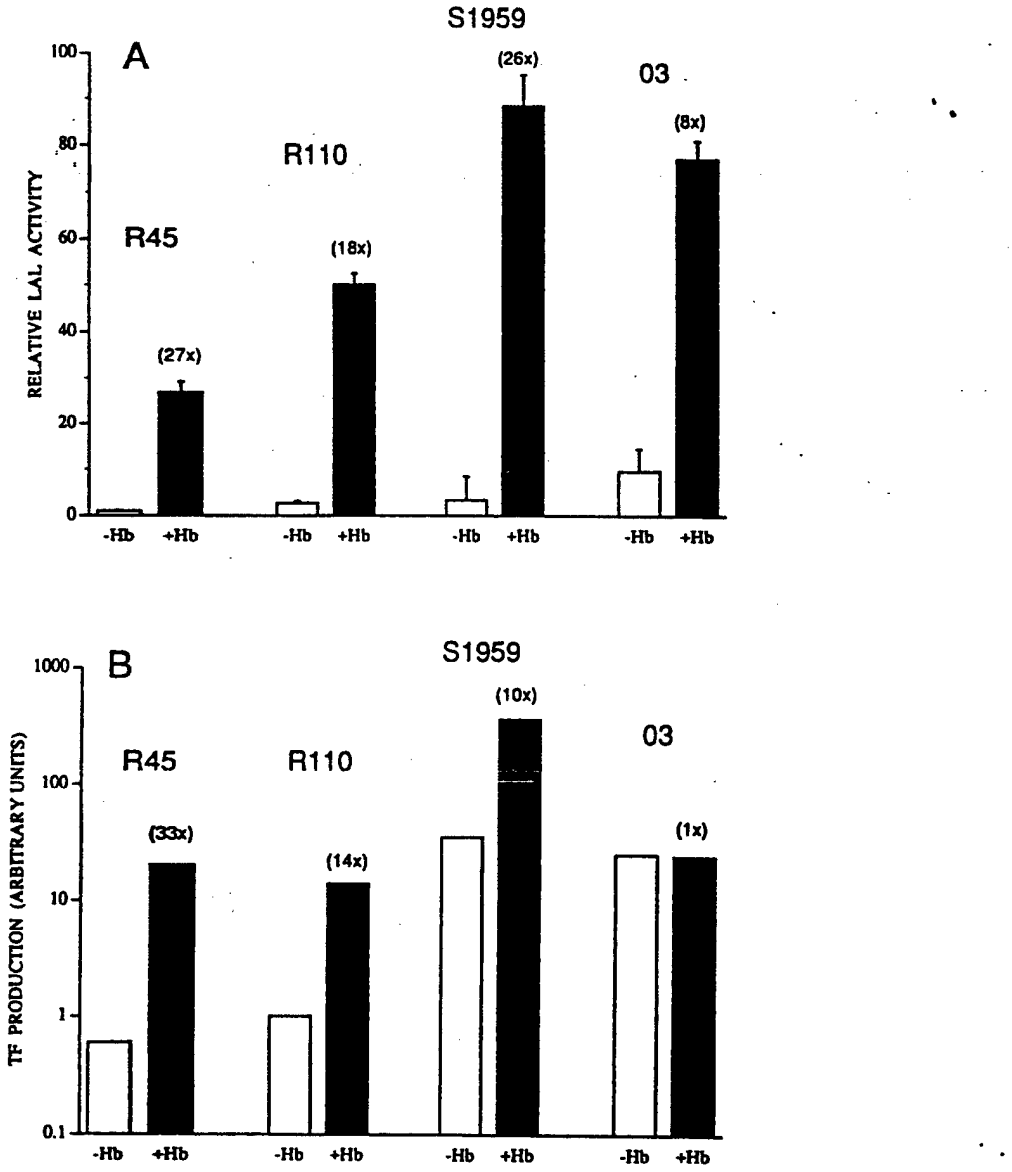

Fig. 1 — Enhancement by Hb of the activation of LAL and production of endothelial cell tissue factor by *Proteus* LPS. **(A)** LAL reactivities of LPS (500 ng/ml) in the absence (–Hb) or presence (+Hb) of αα cross-linked Hb (1 mg/ml) were determined with the chromogenic LAL assay. To determine relative LAL activities, a standard curve of *P. mirabilis* R45 LPS was prepared, which related absorbance to LPS concentration. Using this standard curve, the absorbance for each sample (LPS alone or LPS-Hb) was converted into the equivalent R45 LPS concentration. 500 ng/ml R45 LPS was assigned a relative LAL activity of 1. The fold increase in LAL activity of each LPS, induced by Hb, is indicated in parentheses. Samples were assayed with 8 replicates, and results are expressed as the mean ± 1 SD. **(B)** Cultured human umbilical vein endothelial cells were incubated with *Proteus* LPS (10 µg/ml) in the absence (–Hb) or presence (+Hb) of αα cross-linked Hb (10 mg/ml). TF activities then were determined with a plasma recalcification assay. The fold increase in TF induced by Hb for each LPS is indicated in parentheses. The means of 4 wells are presented.

Molndal, Sweden), utilized in the chromogenic LAL test (below) to measure activation of the LAL proteolytic cascade, was the gift of Dr Petter Friberger, and was reconstituted with pyrogen-free water.

Chromogenic LAL test

Activation of LAL by LPS was quantified with a chromogenic LAL test, as described previously.[15] For most experiments, the LAL was freshly diluted 1:20 in 0.9% NaCl prior to use. For experiments utilizing LBP, undiluted rather than 1:20 LAL was used in order to increase sensitivity to the effects of LPS and LBP.

Endothelial cell tissue factor assay

Production of tissue factor (TF) procoagulant activity from human umbilical vein endothelial cells (obtained from Clonetics Corp., San Diego, CA, USA) was determined as described previously.[30] Briefly, confluent human endothelial cell monolayers in 96-well tissue culture plates were incubated with LPS, in the absence or presence of Hb. After 4 h, TF procoagulant activity was determined with a plasma recalcification assay. TF activity was calculated from the turbidity generated in plasma (the mean from 6–8 replicate wells) based on a standard curve established with dilutions of rabbit brain thromboplastin (Baxter Corporation, Miami, FL, USA). The turbidity generated at 8 min by 1:100 diluted thromboplastin was defined as 1 TF arbitrary unit.

RESULTS

A series of LPS was obtained for the purpose of relating LPS biochemical structure with (1) LPS procoagulant biological activities, and (2) the ability of Hb to enhance these intrinsic LPS activities. In order to examine the role of the hydrophilic core and O chain saccharide on Hb enhancement of LPS activity, 4 *P. mirabilis* LPS were compared for LAL activities in the absence and presence of Hb. These LPS, assayed for relative biological activity in the presence of αα Hb, all demonstrated enhanced LAL activities (Fig. 1A). Similar extents of enhancement also were shown for αα HbCO and HbA_0 (data not shown). For each LPS, enhancement by HSA (another known LPS binding protein[31]) was observed, although the levels of enhancement by Hb were 2- to 3-fold greater than by HSA (data not shown). None of the Hb preparations or HSA activated LAL in the absence of LPS. Because prominent enhancement by αα Hb was observed with the deep rough mutant R45 LPS (containing only lipid A, Kdo and Ara4N), our results indicated that the Hb enhancement effect was a feature of the hydrophobic part of the LPS molecule, and did not require additional hydrophilic saccharide components.

The 4 *P. mirabilis* LPS had different intrinsic biological abilities to activate LAL, as shown in Figure 1A. The parent strain S1959 LPS was 1.3-fold more reactive than R110 LPS, which contains the complete core oligosaccharide, and 3.4 times more reactive than the deep rough mutant R45 LPS. These relative activities suggested that the presence of core oligosaccharide and O-specific polysaccharide moieties increased LAL reactivity. 03 LPS was 2.8 times more reactive than S1959. Since the O-specific polysaccharide chains of the 2 smooth strain LPS, S1959 and 03, differ significantly in their chemical structures, our data also suggested that carbohydrate chemical structure can influence LAL activity.

To ascertain whether enhancement of *Proteus* LPS biological activity by Hb would be observed for another LPS-dependent procoagulant activity, we utilized an assay for tissue factor (TF) production by human endothelial cells. αα Hb enhanced LPS-stimulated TF production by the 2 rough *P. mirabilis* mutant LPS, R45 and R110, and by the smooth LPS S1959, but not by the smooth LPS 03 (Fig. 1B). Endothelial cells alone or in the presence of αα Hb without LPS did not produce measurable TF (data not shown). When S1959 and its mutant LPS R45 and R110 were compared, there was an inverse relationship between relative intrinsic TF production and enhanceability by Hb. Prominent enhancement with R45 LPS provided additional evidence that the Hb enhancement effect was a feature of the lipid A portion of LPS. In both the LAL and TF assays, the activity of 03 LPS was affected the least by αα Hb.

In order to examine the role of lipid A structures in the Hb enhancement effect, biological activities of *S. minnesota* R 595 LPS and chemically modified LPS partial structures were compared in the presence and absence of Hb. Intrinsic biological abilities of these *S. minnesota* LPS to activate LAL, and their respective extents of enhancement by Hb, are shown in Figure 2A. Only the parent 595 LPS demonstrated enhancement of LAL activity by Hb. Similar extents of enhancement also were shown for αα HbCO, HbA_0 and HSA (data not shown). Hydrolysis of even a single fatty acid from R 595 LPS, forming OH37 LPS, completely destroyed the enhancement potential of this LPS. Enhancement of 595 LPS biological activity by αα Hb also was observed in the assay for LPS-induced endothelial cell TF (Fig. 2B). These results confirmed the intrinsic Hb enhanceability of rough 595 LPS observed with LAL activation and the observation that fatty acid hydrolysis resulted in destruction of the Hb enhancement potential of LPS. Interestingly, stimulation of TF by lipid A was prominently enhanced by Hb, whereas LAL activation was not.

Comparison of R 595 intrinsic LAL activities provided data linking LPS structure with LAL biological activity. R 595 LPS, which was relatively insoluble, had modest biological activity (Fig. 2A). Partially deacylated OH37 LPS, which was relatively soluble, readily activated LAL compared to the parent 595 LPS. Lipid A also demonstrated high biological activity. MPL, which was the most insoluble, and OH56 LPS poorly activated LAL. These results indicated that removal of a single 3-hydroxytetradecanoyl acid residue

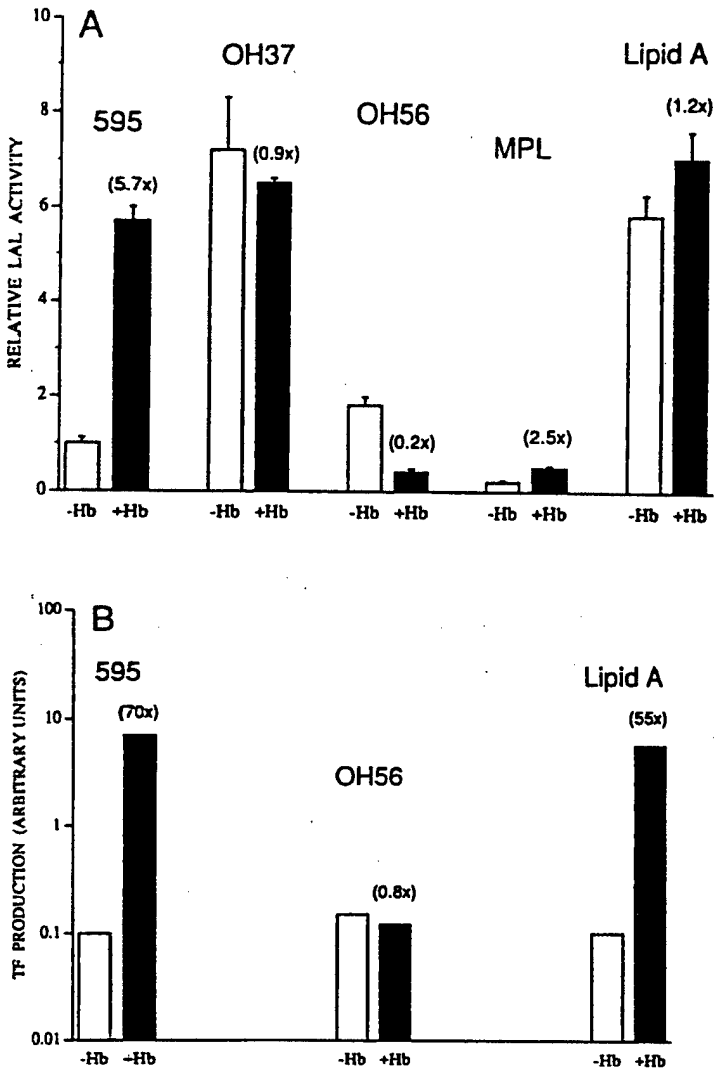

Fig. 2 — Enhancement by Hb of the activation of LAL and production of endothelial cell tissue factor by *Salmonella* LPS. (A) LAL reactivities of LPS (500 ng/ml) in the absence (–Hb) or presence (+Hb) of αα cross-linked Hb (1 mg/ml) were determined with the chromogenic LAL assay. To determine relative LAL activities, a standard curve of parent *S. minnesota* R 595 LPS was prepared, which related absorbance to LPS concentration. Using this standard curve, the absorbance for each sample (LPS alone or LPS-Hb) was converted into the equivalent R 595 LPS concentration. 500 ng/ml R 595 LPS was assigned a relative LAL activity of 1. The fold increase in LAL activity of each LPS, induced by Hb, is indicated in parentheses. Samples were assayed with 8 replicates, and results are expressed as the mean ± 1 SD. (B) Cultured human umbilical vein endothelial cells were incubated with *Salmonella* LPS (10 μg/ml) in the absence (–Hb) or presence (+Hb) of αα cross-linked Hb (10 mg/ml). TF activities then were determined with a plasma recalcification assay. The fold increase in TF induced by Hb for each LPS is indicated in parentheses. The means of 4 wells are presented.

from the reducing glucosamine of the lipid A moiety of R 595 LPS, or removal of the Kdo molecule from the core region, generated modified LPS molecules which could more efficiently interact with LAL, whereas further deacylation of the R 595 LPS to OH56 LPS resulted in a modified LPS with biological activity similar to that of the parent LPS. Therefore, the core Kdo and at least one of the fatty acid residues of lipid A apparently are not crucial for LAL activation. There was a dramatic loss of LAL reactivity after partial dephosphorylation of LPS to generate MPL, indicating that the phosphate group of lipid A was critical for LAL reactivity or that biologic activity was dependent upon adequate solubility,

Many of the LPS preparations studied had poor aqueous solubility and were visually turbid (especially *S. minnesota* 595 LPS, lipid A and MPL, and *P. mirabilis* R110). Hb enhancement of LPS biological activity was a prominent feature of some of these LPS and partial structures, suggesting that a possible mechanism for the Hb enhancement effect was via increased LPS solubility. Therefore, we compared turbidity and the LAL biological activity of these LPS in the absence and presence of Hb. With increasing concentrations of ααHb, *P. mirabilis* R110 and *S. minnesota* 595 LPS each demonstrated a concomitant progressive decrease in turbidity and increase in LAL biological activity (Fig. 3). A similar but smaller change in turbidity, and an increase in LPS biological activity, were observed with lipid A. MPL turbidity was shown to increase rather

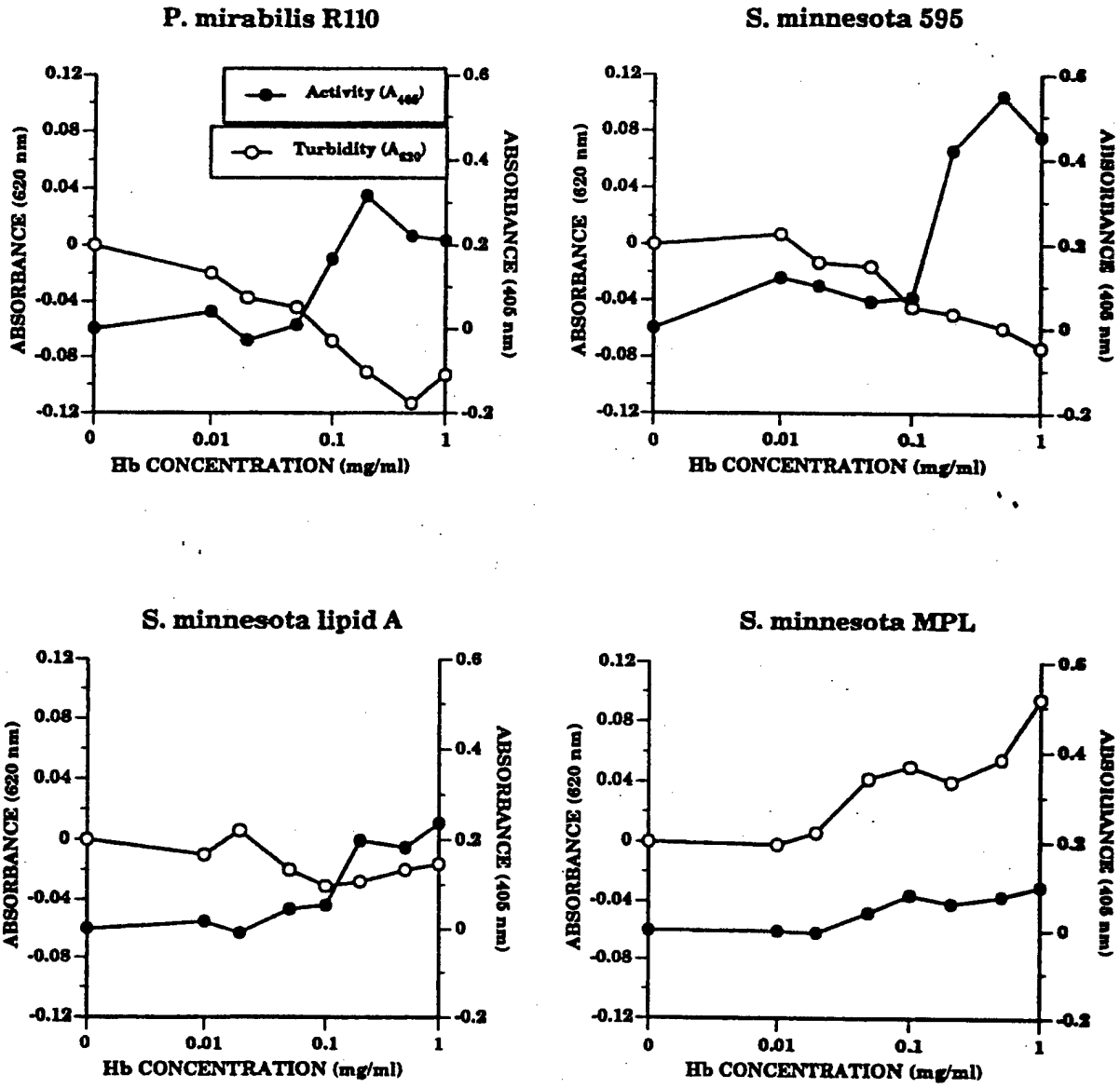

Fig. 3 — Turbidity and biologic activities of LPS in the absence and presence of Hb. Various concentrations of αα cross-linked Hb (from 0.01 to 1.0 mg/ml) were added to LPS (final concentration, 1 mg/ml) in microtiter plate wells and absorbances were measured at 620 nm. The turbidity of each LPS (absorbance at 620 nm) in the absence of Hb has been designated as 0, and the change in absorbance induced by Hb is shown. Absorbances due to Hb have been subtracted. Actual baseline LPS absorbances were as follows: *P. mirabilis* R110, 0.21; *S. minnesota* R 595, 0.12; *S. minnesota* lipid A, 0.61; and *S. minnesota* MPL, 0.65. LAL then was added to each well and chromogenic activities determined at 405 nm.

than decrease with addition of ααHb, and biological activity was unchanged. In a control experiment using the same LPS, HSA demonstrated no effect on LPS turbidity (data not shown). In this experiment, HSA enhanced the biological activity of R110 and had little effect on the other LPS.

In order to further establish the generalized nature of the Hb enhancement effect, we studied the effect of ααHb on biological activities of several other LPS, including LPS from different bacterial species. Prominent, and identical, extents of enhancement by both ααHb and ααHbCO in the LAL assay were shown with 3 defined salts of *E. coli* O26:B6 (smooth LPS), i.e. the calcium, sodium and triethylamine forms, suggesting that the specific cations bound to LPS did not influence the Hb enhancement process. *E. coli* O26:B6 LPS-induced endothelial cell TF also was enhanced by ααHb (mean of 13-fold enhancement in 7 experiments, range 8- to 26-fold enhancement). Finally, enhancement of LPS biological activity was demonstrated with a smooth *Salmonella* LPS (*S. abortus equi*) and a rough *E. coli* LPS (Re F515), but was not observed with non-toxic *R. spheroides*, *R. capsulatus* and *Rh. viridis* LPS (data for the above not shown).

To compare the enhancement ability of Hb with other plasma proteins, LAL activation by *S. minnesota* R 595 LPS was determined in the absence or presence of Hb and several other previously identified LPS binding protein. Because of limited availability of LPS binding protein, the comparison of Hb and LPS binding

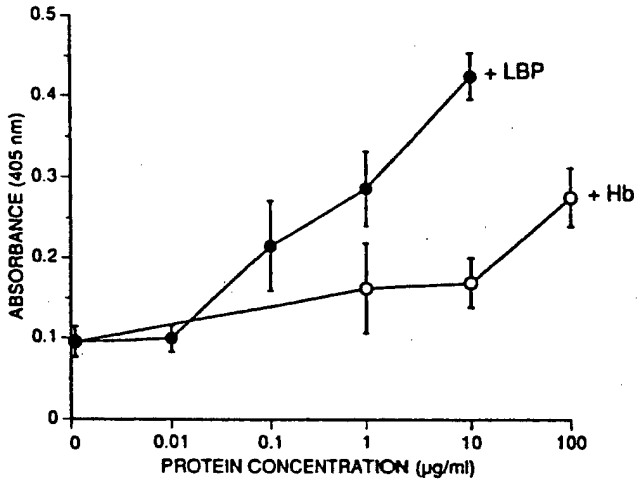

Fig. 4 — Comparison of Hb and LBP for ability to enhance LPS activation of LAL. *S. minnesota* R 595 LPS (10 pg/ml), in the absence or presence of αα cross-linked Hb in concentrations ranging from 1 to 100 µg/ml) or LBP (lipopolysaccharide binding protein) in concentrations ranging from 0.01 to 10 µg/ml), was assayed with the chromogenic LAL test using undiluted LAL. Samples were assayed in triplicate, and results are expressed as the mean ± 1 SD.

protein was performed using undiluted LAL, as described in Methods. In this experiment, the biological activity of 10 pg/ml LPS was clearly initially increased in the presence of 0.1 µg/ml LBP or 1–10 µg/ml ααHb (Fig. 4). Throughout almost the entire range of ααHb concentrations tested for enhancement of 595 LPS biological activity, equivalent enhancement was produced by LBP at a much lower protein concentration.

Comparisons also were made between HbA_0 and HSA, IgG or transferrin using the standard chromogenic assay (Fig. 5). HbA_0 and albumin enhanced *S. minnesota* R 595 LPS LAL reactivity in a dose-dependent manner, although the enhancement effect of HbA_0 was detectable at 0.03 mg/ml, whereas equivalent enhancement of 595 LPS by albumin was only observed at > 0.25 mg/ml protein. In contrast to LPS enhancement by HbA_0 and albumin, neither IgG nor transferrin had any demonstrable effect on 595 LPS LAL reactivity. Similar results were obtained using ααHb in comparison with albumin, IgG and transferrin (data not shown). Therefore, of the 5 human proteins studied, LBP and Hb were effective at low concentrations (≤ 0.03 mg/ml) in enhancing LAL activation by LPS, whereas albumin, IgG and transferrin had little or no such capability at comparable concentrations (compare Figs 4 and 5). The lack of effect of IgG on LAL activation by LPS is in contrast to our previous study which demonstrated inhibition by IgG.[16] However, subsequent investigation has demonstrated that the inhibitory activity detected in the previously studied preparation of IgG was attributable to maltose used as a stabilizer rather than the IgG per se (personal observation).

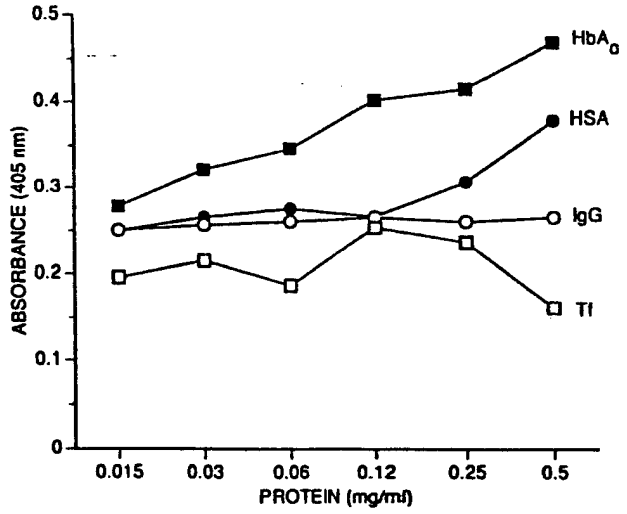

Fig. 5 — Influence of plasma proteins on the ability of *S. minnesota* R 595 LPS to activate LAL. *S. minnesota* R 595 LPS (30 ng/ml) was incubated for 5 min at 37°C with native HbA_0, HSA, human IgG or human transferrin (Tf) (each at concentrations from 0.015 to 0.5 mg/ml), and chromogenic LAL assays were performed. Absorbance at 405 nm for LPS alone was 0.24. Samples were assayed in duplicate, and mean values are presented.

Since IgG did not enhance the LAL reaction, it was possible to investigate the potential of this known LPS binding protein to inhibit LPS enhancement by Hb. Therefore, we studied whether the presence of IgG would prevent enhancement of LPS-induced LAL activation by Hb. *S. minnesota* R 595 LPS was preincubated with either IgG or HbA_0, followed by subsequent incubation with the other protein. Addition of IgG to a preincubated mixture of LPS and HbA_0 slightly decreased Hb-induced enhancement at the lower concentrations of LPS studied, but had no effect at higher LPS concentrations (data not shown). Addition of HbA_0 to a preincubated mixture of LPS and IgG similarly demonstrated partial inhibition of the Hb effect at the lower LPS concentrations, but no inhibition at higher LPS concentrations. These results are consistent with competition between Hb and IgG for LPS, but with Hb demonstrating the greater affinity.

DISCUSSION

LAL reactivities of our battery of LPS were examined in the presence of Hb in order to define the constituents of the LPS molecule required for interaction with Hb. ααHb enhanced the biological activity of each of the *Proteus* LPS tested, including R45 LPS which contains only lipid A, Kdo and 4-amino-arabinose residues.[19,20] This suggested that the hydrophobic part of LPS, i.e. lipid A, was responsible for the interaction with Hb and the resultant increase of biological activity of LPS. ααHb also enhanced rough LPS-stimulated tissue factor production from endothelial cells, and thus provided further support for the conclusion that components of lipid A are required for interaction with Hb. Lipid A chemical moieties then were studied utilizing *S. minnesota* R 595 LPS and its chemically modified derivatives in the presence of Hb. Whereas intact R 595 LPS

demonstrated prominent enhancement of its biological activity by ααHb, singly deacylated OH37 LPS and multiply deacylated OH56 LPS failed to be enhanced in the LAL assay. Similarly, OH56 LPS failed to be enhanced in the endothelial cell tissue factor assay. These results suggested that the binding process between Hb and LPS, that leads to enhancement, may involve an important interaction of this protein with the ester-linked fatty acyl residues of lipid A. Our studies demonstrated a similar necessity for lipid A fatty acids for the increase in LPS biological activity induced by human albumin, an avid fatty acid binding protein. Enhancement of MPL (lacking the phosphate residue of the reducing glucosamine) or lipid A (lacking the Kdo residues of the core) was substantially less than that of the parent R 595 LPS, but was not totally absent as for the deacylated LPS (Fig. 2). These results suggested that phosphate and Kdo residues are less important in the enhancement process than the ester-linked fatty acids.

In general, those LPS with poor solubility in water (i.e. *P. mirabilis* R45, *S. minnesota* R 595 and its MPL derivative) were more effectively enhanced by Hb than those with better solubility (i.e. *S. minnesota* R 595 derivatives OH37 and OH56). This suggested that Hb has a more important detergent-like, disaggregating effect on those LPS with poor initial solubility. This conclusion is supported by our previous observations that Hb decreases the apparent molecular weight of LPS.[15] In further support of this potential mechanism for Hb enhancement of LPS biological activity, we demonstrated that ααHb decreased the turbidity (i.e. increased the solubility) of the poorly soluble LPS. Disaggregation of LPS micelles, resulting in enhanced LAL activity of LPS, similarly has been proposed as the reason for the increased potency of LPS in the presence of transferrin.[32] It is interesting that enhancement of LPS biological activity by albumin in our studies was not associated with decreased LPS turbidity, suggesting that Hb and albumin enhance LPS activity by different mechanisms.

In contrast to the effects of chemical modification of LPS on their LAL biological activities and the process of Hb enhancement, three distinct salt forms of *E. coli* O26 LPS (Na, Ca and triethylamine) had similar LAL reactivities and demonstrated pronounced and identical enhancement by ααHb. These results are in agreement with previous observations that *Limulus* gelation activities were similar for a variety of electrodialyzed defined salts of *S. abortus equi* LPS.[33]

Equivalent extents of Hb enhancement of LPS activity were observed with native HbA_0 as with ααHb, demonstrating that this was an intrinsic property of Hb. Enhancement of bioavailability of LPS with cross-linked Hb indicated that dissociation of the Hb tetramer was not involved in this effect. Because equivalent enhancement results also were obtained with ααHbCO, the mechanism of the enhancement effect did not require the production of methemoglobin, and enhancement was not a result Hb oxidation and denaturation. However, we have observed that LPS in high concentration (0.5–1 mg/ml), is capable of facilitating Hb denaturation (unpublished observations).

Our comparisons of the relative LAL activities of *Proteus* and *Salmonella* LPS specifically demonstrated the contribution of the Kdo residues of the core, and the fatty acyl and phosphoryl residues of lipid A, to LPS intrinsic biological activity. The alterations in LAL potencies of chemically modified 595 LPS may have resulted from changes in solubility in aqueous solutions of the modified LPS as well as the removal of residues important for LAL activation. In addition, heterogeneity of both native and chemically degraded *S. minnesota* 595 LPS may also influence LAL reactivity. These observations are consistent with previous studies concluding that *Salmonella* and *Escherichia* lipid A require at least one phosphate residue and two fatty acyl chains for activation of LAL,[34–36] and that chemically synthesized MPL[37] or highly purified *E. coli* MPL[34] have reduced LAL potency. Interestingly, 3-O-deacylated LPS such as the *S. minnesota* OH37 LPS we prepared is known to possess a large and energetically unfavorable cavity in the interior part of the LPS molecule which results in a conformational reorganization of lipid A,[25] and in our experiments this chemical modification resulted in increased biological activity in the LAL assay. In contrast, similar 3-O-deacylation of *E. coli* J5 and *S. typhimurium* LPS has been shown to result in LPS with unaltered LAL activity and mitogenicity, and with attenuated rabbit pyrogenicity and decreased chick embryo and mouse lethality,[25] and the removal of secondary acyl chains from the lipid A moiety of LPS by leukocyte acyloxyacyl hydrolase has been reported previously to reduce LPS LAL potency.[38] It is not known whether these differences are related to the chemical composition of the LPS tested or to differences in the biological assays utilized. The enhancement of LPS biological activity by Hb also requires these lipid A moieties and in addition, Kdo residues, and likely represents a mechanism that involves the disaggregation and solubilization of LPS by Hb. Interestingly, Hb did not enhance LAL activity of non-enterobacterial, non-toxic *Rhodobacter* and *Rhodopseudomonas* LPS. This may indicate that a 'toxic conformation'[39] of LPS, not present in non-toxic LPS,[40] is required for Hb enhancement. It is likely that the phenomenon of Hb enhancement of LPS biological activity has physiologic and clinical relevance because of the difficulty in production of Hb without substantial endotoxin contamination,[16] the likelihood that Hb would be transfused into trauma patients with concomitant endotoxemia, and the potential for hemoglobinemia (secondary to intravascular hemolysis during sepsis) to augment the deleterious effects of endotoxemia.

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Hemoglobin Enhances the Production of Tissue Factor by Endothelial Cells in Response to Bacterial Endotoxin

By Robert I. Roth

Human endothelial cells respond to bacterial endotoxin (lipopolysaccharide [LPS]) with changes that transform the endothelium into a surface with prominent procoagulant properties. Production of tissue factor (TF) in response to LPS is a major alteration that favors coagulation. Biologic activities of LPS have previously been shown to be enhanced by the presence of hemoglobin. Therefore, the ability of human hemoglobin (Hb) to modulate TF production by cultured human umbilical vein endothelial cells (HUVEC) was investigated. Cell-free Hb (10 mg/mL), either purified native (HbA_o) or chemically cross-linked ($\alpha\alpha$Hb), was incubated with LPS (0.1 μg/mL), and the mixtures then were added to HUVEC in culture. TF activity was quantified with a clotting assay and TF protein was measured with an enzyme-linked immunosorbant assay. Hb preparations greatly enhanced the production of TF activity (11- to 25-fold greater than TF produced by HUVEC alone) compared with minimal TF activity generated by LPS alone (only twofold greater than HUVEC alone). The enhancement of LPS-induced TF activity was Hb concentration-dependent over a range of 1 to 100 mg/mL. Cross-linked $\alpha\alpha$Hb also greatly enhanced the production of TF protein compared with TF protein generated by LPS alone (12-fold greater v 3.5-fold greater than HUVEC alone, respectively). The enhancement of LPS-induced TF protein was Hb concentration-dependent over a range of 0.1 to 2 mg/mL. Enhancement of TF activity by Hb required new protein synthesis. These results show that human Hb can augment the ability of LPS to induce endothelial cell TF and suggest that hemolysis associated with disseminated intravascular coagulation during sepsis may further stimulate coagulation. In addition, these results suggest a potential mechanism for generalized thrombosis in animals that has been associated with the infusion of cell-free Hb for resuscitation.

HUMAN ENDOTHELIAL CELLS have a major role in the control of hemostasis. Under normal conditions, the endothelium provides an antithrombotic barrier. Contributing to thromboresistance are the expression of anticoagulant factors such as protein S, thrombomodulin and plasminogen activators,[1,2] presentation of heparin-like molecules,[3] and the inhibition of platelet aggregation by prostacyclin and endothelium-derived relaxing factor.[4] Recently, an inhibitor of the contact activation of coagulation via Hageman factor also has been described.[5] During gram-negative bacterial sepsis, the presence of bacterial lipopolysaccharide (LPS) results in prominent changes involving the endothelium. Endothelial cells exposed to LPS show overall prothrombotic properties, principally by the synthesis and expression of tissue factor (TF),[6,7] the upregulation of plasminogen activator inhibitor,[8] the down regulation of thrombomodulin, and the inhibition of factor C activation.[7] Leukocyte adhesion is enhanced in the presence of LPS,[9,10] and when human umbilical vein endothelial cells (HUVEC) are incubated with thrombin, a coagulation protease that is commonly generated during endotoxemia, adherence of platelets to HUVEC is increased.[11] These endothelial cell changes in response to LPS and the subsequent prothrombotic activities of the endothelium contribute to the multiple organ failure that is one of the prominent pathologic consequences of gram-negative sepsis.

Cell-free hemoglobin (Hb) can be released from erythrocytes during sepsis as a result of coagulation-mediated intravascular hemolysis or bacterial hemolysin activity.[12,13] Plasma Hb levels were reported to be 1 to 2 mg/mL in patients with intravascular hemolysis,[14] and plasma Hb levels up to 2 mg/mL have been reported in rabbits with enzyme-mediated hemolysis[15] or endotoxin-mediated intravascular hemolysis.[16] In these studies, released Hb was in excess of the binding capacity of haptoglobin and was detected as free Hb for several hours. Therefore, LPS and considerable concentrations of Hb may coexist in the blood stream during pathologic conditions, and in previous studies in our laboratory, it has been shown that the two molecules form Hb-LPS complexes.[17] In vitro studies and in vivo observations have suggested that significant pathophysiologic consequences result from the biochemical interaction(s) between Hb and LPS. Hb has been shown in vitro to enhance LPS-initiated activation of coagulation[18] and to stimulate production of mononuclear cell TF.[19] In vivo, synergism of the toxicities of Hb and LPS (eg, synergistic activation of coagulation[20,21] and complement[21] cascades) has been shown, suggesting that enhancement of LPS activity by Hb may contribute to the observed thrombosis, ischemic damage, and multiple organ failure associated with Hb infusion in animals. Therefore, Hb-LPS interactions, resulting from sepsis-mediated hemolysis, may potentiate the deleterious effects of LPS. In addition, the interaction between Hb and LPS in vivo, with resultant synergism of their pathophysiologic effects, constitutes a major potential limitation in the use of Hb-based oxygen carriers for resuscitation.[22] In the present

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study, the effect of Hb on the production of TF by endothelial cells in response to bacterial endotoxin was examined.

MATERIALS AND METHODS

Reagents. Actinomycin D and cycloheximide were obtained from Sigma (St Louis, MO). Sterile tissue-culture plasticware was obtained from Becton Dickinson (Mountain View, CA).

Hb. Purified human hemoglobin A_0 (HbA_0), prepared by ion exchange high pressure liquid chromatography of purified human Hb, as described previously,[23] was provided by collaborators at the Blood Research Division of the Letterman Army Institute of Research (BRD/LAIR; San Francisco, CA). Human cross-linked cell-free hemoglobin ($\alpha\alpha$Hb), covalently cross-linked between the two α-chains with bis(3,5-dibromosalicyl) fumarate as described previously,[24] also was provided by collaborators at BRD/LAIR. These Hb preparations contained less than 0.4 EU/mL endotoxin (referenced to *Escherichia coli* LPS B, O55:B5; Difco Laboratories, Detroit, MI), as determined by the Limulus amebocyte lysate test,[25] and did not contain demonstrable erythrocyte stroma, as shown by phosphorus analysis and reverse-phase high pressure liquid chromatography. Hb concentrations were determined spectrophotometrically.

Endotoxin. *E coli* O26:B6 (W) LPS was purchased from Difco Laboratories.

HUVEC. HUVEC and endothelial cell culture media containing 2% fetal bovine serum, 10 ng/mL epidermal growth factor (EGF) and 1 ng/mL hydrocortisone were purchased from Clonetics (San Diego, CA). HUVEC were plated in 96-well microtiter plates (Nunclon; Applied Scientific, South San Francisco, CA) at a seeding density of 3,500 cells/cm^2 (5,000 cells/well) and were grown to confluency at 37°C and 5% CO_2. HUVEC were used at less than 6 passages.

TF procoagulant assay. TF activity was quantified with a plasma recalcification assay. Confluent HUVEC monolayers in 96-well plates were incubated for 4 hours at 37°C with 10 μL *E coli* LPS alone, HbA_0 or $\alpha\alpha$Hb alone, or Hb-LPS mixtures in 90 μL media/well. Standard incubations were conducted using 10 mg/mL Hb. Experiments were performed with 6 to 8 replicate wells. Wells were then washed with media (×3) and the HUVEC were freeze-thawed twice and sonicated in 50 μL phosphate-buffered saline for 2 minutes at room temperature. To each well then was added 50 μL normal human citrated plasma and 50 μL calcium (25 mmol/L), and after 8 minutes, turbidity was measured at 340 nm in a 37°C temperature-controlled plate reader (Kinetic-QLC; Whittaker Bioproducts Inc, Walkersville, MD). TF activity was calculated from the turbidity generated in plasma (the mean from 6 to 8 replicate wells) by a standard curve established with dilutions of rabbit brain thromboplastin (Baxter Corporation, Miami, FL). The turbidity generated at 8 minutes by 1:100 diluted thromboplastin was defined as 1 TF arbitrary unit.

TF protein assay. Confluent HUVEC monolayers in 96-well plates were incubated for 4 hours at 37°C with 10 μL *E coli* LPS alone, HbA_0 or $\alpha\alpha$Hb alone, or Hb-LPS mixtures in 90 μL media/well. Standard incubations were conducted using 2 mg/mL Hb. Experiments were performed with 6 to 8 replicate wells. Wells were then washed with media (×3), and the HUVEC were freeze-thawed twice and sonicated in 50 μL phosphate buffered saline; 10 μL Triton X-100 was added (final concentration, 1%), and incubations were continued overnight at 4°C. The solutions were then removed from the plates and centrifuged for 15 minutes (Microfuge B; Beckman Instruments, Inc, Palo Alto, CA). TF was quantified by enzyme-linked immunosorbant assay (ELISA) using a murine antihuman TF monoclonal antibody (Imubind; generously provided by American Diagnostica, Inc, Greenwich, CT) according to the manufacturer's directions.

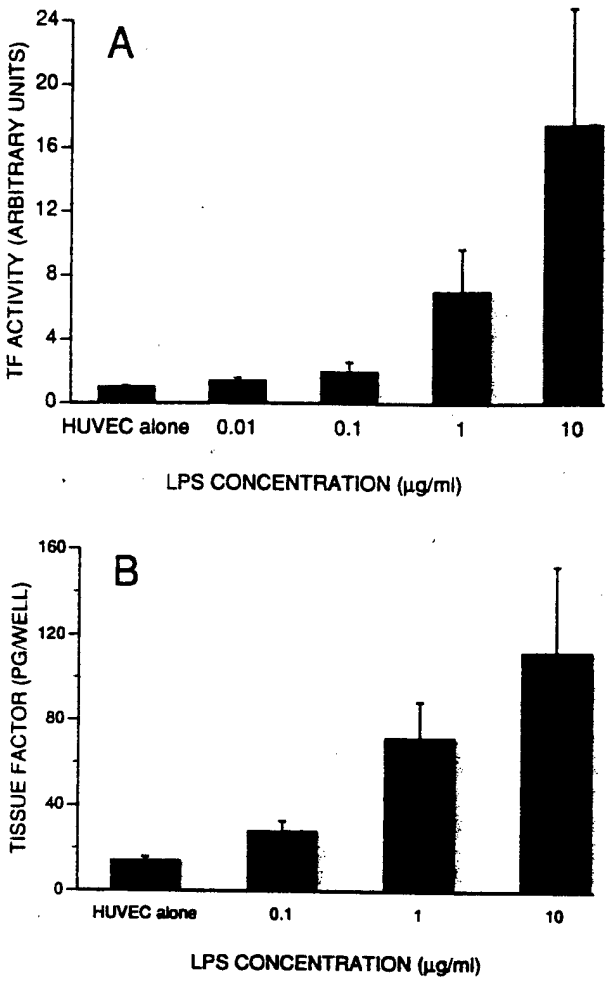

Fig 1. TF produced by HUVEC in response to LPS is shown. Various concentrations of *E coli* LPS were incubated with HUVEC for 4 hours, and TF activities (A) and TF protein concentrations (B) were determined. (A) TF activities, determined with a plasma recalcification assay, were normalized to 1 for TF production by HUVEC alone. The means and 1 SD of 8 tissue culture-plate wells are presented. (B) TF production, expressed as picograms of TF per tissue culture-plate well, was determined with an ELISA assay. The means and 1 SD of 6 wells are presented.

RESULTS

HUVEC contained undetectable or very low TF activity (<0.1 TF unit), as determined by the plasma recalcification assay. After incubation with *E coli* LPS, a concentration-dependent and time-dependent stimulation of HUVEC TF production was observed. In a representative experiment, stimulation of TF activity ranged from 1.4-fold over baseline, after 4-hour incubation with 0.01 μg/mL LPS, to 17.5-fold over baseline, after incubation with 10 μg/mL LPS (Fig 1A). At 100 μg/mL or 1 mg/mL LPS, TF production in some experiments was less than the TF produced by 10 μg/mL LPS and, in other experiments, was equivalent. The increase in TF was detectable after 2 hours of incubation with LPS and became maximal between 4 and 6 hours (data not

Table 1. Relative Procoagulant Activities of Chemically Different Glycolipids

Lipid A*	Deep Rough LPS Mutant†	Rough LPS Mutant‡	Rough LPS Mutant§	Smooth LPS	Smooth LPS¶
1	3	8	18	4	6

Various types of purified LPS were incubated with HUVEC for 4 hours, and TF activities were determined. The LPS that elicited the least TF response by the HUVEC (*S minnesota* lipid A) was assigned a relative procoagulant activity of 1, and the biologic activities of the other LPSs are presented in comparison to lipid A.

* *S minnesota* lipid A.

† *S minnesota* 595.

‡ *Protens mirabilis* R110.

§ *P mirabilis* R45.

P mirabilis S1959.

¶ *E coli* O55:B5.

shown). Similar LPS concentration-dependent TF responses were detected in 8 independent experiments; the maximal TF activities from LPS-treated HUVEC (achieved with 10 μg/mL LPS) were from 7 to 28 times greater (mean, 16 times greater) than the TF activity of untreated HUVEC. TF responses varied considerably when different glycolipids were examined (Table 1). A low TF response was observed with *S minnesota* lipid A (which consists of a diglucosamine backbone and seven fatty acyl chains) compared with other LPSs which contained additional saccharide moieties, suggesting that O-chain and core carbohydrates are important for the HUVEC response. We also quantified HUVEC TF protein by a sensitive ELISA assay. In an experiment representative of 6 independent studies, TF protein increased in an LPS concentration-dependent manner from 17 pg/well in untreated HUVEC to 116 pg/well in HUVEC after 4 hr incubation with 10 μg/ml LPS (Fig 1B). Total protein per well was unchanged at 41 ± 4 μg/well. In each of the 6 studies, LPS-treated HUVEC showed both increased TF functional activity and increased antigenic concentrations of TF protein.

To further demonstrate that the TF activity induced by LPS resulted from newly formed protein rather than by a process of enhanced catalytic activity by preexisting TF protein, we examined the effect of protein synthesis inhibitors. LPS-stimulated TF production was completely inhibited by actinomycin D or cycloheximide (Fig 2), providing additional evidence that the cellular procoagulant response to LPS required new protein synthesis.

To test the effect of Hb on the production of HUVEC TF in response to LPS, two Hb preparations were investigated. HbA_o was used because this preparation was native Hb and would be potentially available to interact with the endothelium during in vivo hemolysis of erythrocytes. $\alpha\alpha$Hb was used because this chemically stabilized preparation is not susceptible to dissociation of the α-chains of the Hb tetramer[26] and is a form of Hb that is presently being developed as a cell-free blood substitute. Hb (10 mg/mL) was first incubated with LPS for 30 minutes at 37°C, and the mixtures then were added to HUVEC. A low concentration of LPS

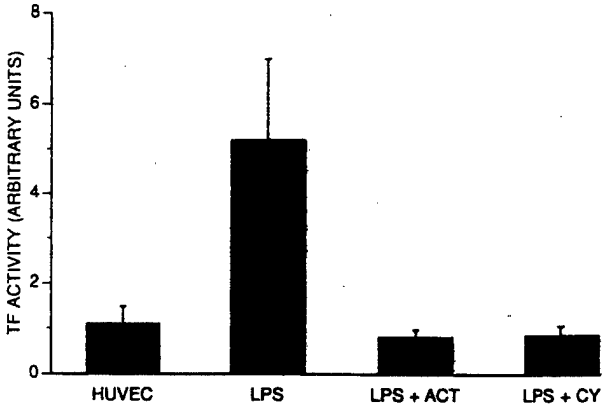

Fig 2. Effect of protein synthesis inhibitors on production of TF by HUVEC in response to LPS is shown. HUVEC were incubated with *E coli* LPS (1 μg/mL) for 4 hours in the absence or presence of actinomycin D (ACT) or cycloheximide (CY; each 10 μg/mL), and TF activities then were determined with a plasma recalcification assay. The means and 1 SD of 8 tissue culture-plate wells are presented.

was used (0.1 μg/mL) so that only modest TF production by HUVEC was generated in response to LPS alone. At this concentration, HUVEC incubated with LPS generated only 2.5-fold greater TF activity than unstimulated HUVEC (Fig 3). HbA_o or $\alpha\alpha$Hb alone (10 mg/mL each) did not demonstrably increase the very low levels of TF activity produced by HUVEC in the absence of LPS. In contrast, stimulation of TF production from HUVEC was 11-fold increased in the presence of the LPS-HbA_o mixture and was 25-fold increased in the presence of the LPS-$\alpha\alpha$Hb mixture. Preincubation of Hb and LPS longer than 30 minutes before their addition to HUVEC did not alter this response.

The enhanced TF production in the presence of various concentrations of Hb was concentration-dependent, as is

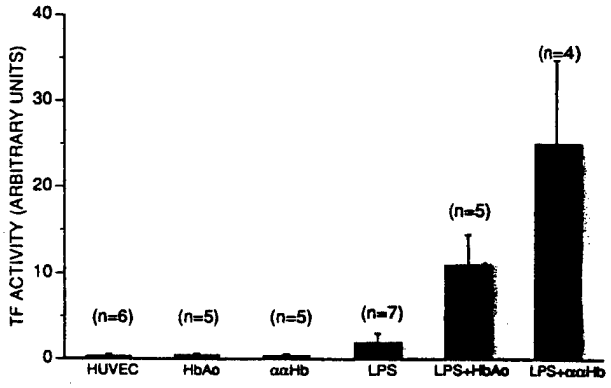

Fig 3. TF activity produced by cultured HUVEC in the presence of LPS and LPS-Hb mixtures is shown. HUVEC were incubated with *E coli* LPS (0.1 μg/mL) for 4 hours in the absence or presence of HbA_o or $\alpha\alpha$Hb (10 mg/mL each). TF activities were determined with a plasma recalcification assay. The means and 1 SD of 4 to 7 independent experiments (n) are presented. For each independent experiment, TF activity was determined from the average of 6 tissue culture wells.

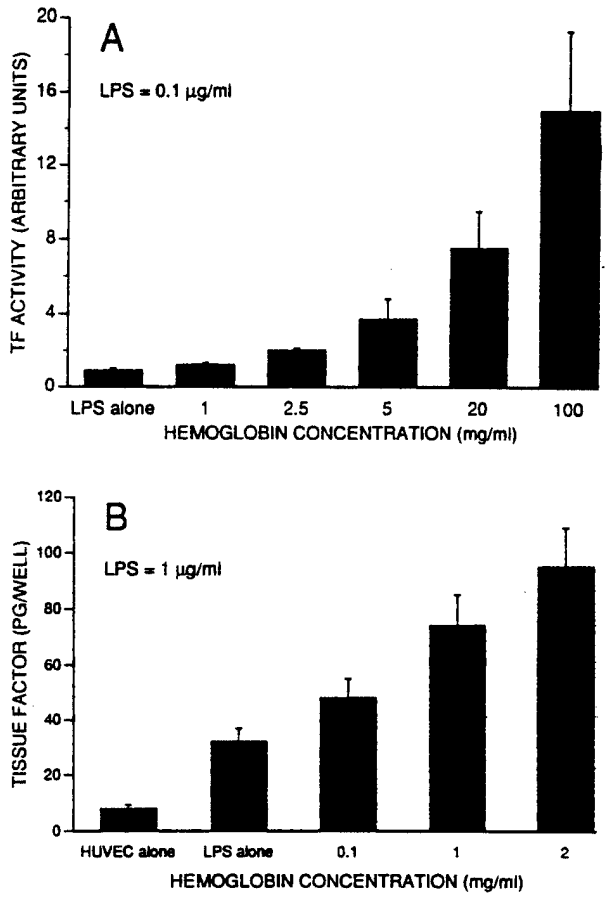

Fig 4. Enhanced production of TF by HUVEC in the presence of LPS-Hb is shown. (A) *E coli* LPS (0.1 μg/mL) was incubated with various concentrations of HbA_o and the mixtures, or LPS alone, then were added to HUVEC in culture. After incubation for 4 hours, TF activity was quantified by a plasma recalcification assay. TF activities were normalized to 1 for TF production by HUVEC in response to LPS alone. (B) HUVEC were incubated with *E coli* LPS (1 μg/mL) alone or with LPS in the presence of various concentrations of αα Hb. TF production, expressed as picograms of TF per tissue culture-plate well, was determined with an ELISA assay. The means and 1 SD of 6 wells are presented.

shown for HbA_o (Fig 4A). A similar response to increasing concentrations of HbA_o was observed when experiments were performed with αα Hb (data not shown). Supernatants of the HUVEC cultures did not contain detectable TF activity, even after incubation with LPS or LPS-Hb mixtures (data not shown). ELISAs showed Hb concentration-dependent synthesis of new TF protein in response to LPS-Hb mixtures (Fig 4B). In this representative experiment, TF protein production was stimulated 3.9-fold over baseline by 1 μg/mL LPS (in the absence of Hb) and 11.8-fold over baseline in the presence of LPS and 2 mg/mL αα Hb. This represents a threefold enhancement in TF protein production because of αα Hb. In 4 independent experiments, maximum TF concentrations (pg/well) produced in response to Hb-LPS mixtures were 2.1- to 6.5-fold greater (mean, 3.7-fold) than the concentrations of TF protein produced in response to LPS alone.

To determine whether the enhancement of LPS-elicited TF production in the presence of Hb was the result of new TF protein production, HUVEC were incubated with LPS and Hb in the presence of actinomycin D or cycloheximide. Each of the protein synthesis inhibitors totally prevented the generation of TF activity, indicating that the mechanism for the Hb enhancement process involved new protein synthesis (Fig 5).

DISCUSSION

Despite the extensive existing knowledge of the structure of human Hb and its function within the erythrocyte, relatively little is known about pathophysiologic interactions involving extraerythrocytic Hb and other blood elements and host tissues. Clinical evidence of renal, vascular, and reticuloendothelial system damage during hemolytic episodes has suggested that extraerythrocytic Hb (and, in particular, Hb breakdown products such as hematin) can be toxic.[27] However, the direct effects of Hb have been difficult to determine because the contribution to the observed toxicities from erythrocyte membrane components is also felt to be of major importance.[21,28] Similarly, in determining the mechanism of organ toxicity associated with LPS-mediated intravascular coagulation with resultant hemolysis, it is difficult to distinguish the contribution of Hb to the observed deleterious effects from the contribution by LPS.[20,21,28] The elucidation of LPS-mediated toxicity itself is difficult because of the high potency and the protean biologic activities of LPS. The potential for LPS and cell-free Hb to interact in the blood stream adds additional complexity to an understanding of the consequences of extraerythrocytic Hb.

The current efforts to develop cell-free Hb as a transfusion product have allowed detailed investigation of the interactions between Hb and LPS. Previously, we had shown that there is a biologically significant interaction between Hb and

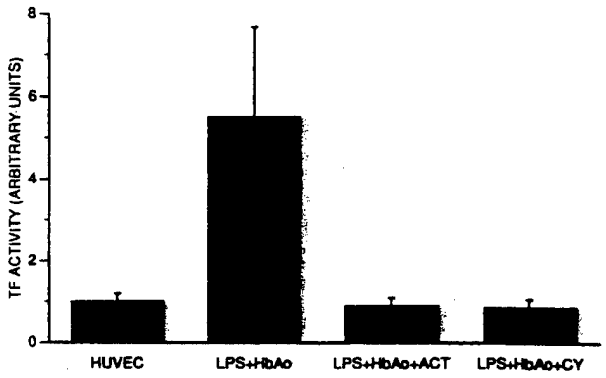

Fig 5. Effect of protein synthesis inhibitors on production of TF by HUVEC in response to LPS-Hb mixtures is shown. *E coli* LPS (1 μg/mL) was incubated with HbA_o (10 mg/mL), and the mixtures, or LPS alone, then were incubated with HUVEC for 4 hours in the absence or presence of actinomycin D (ACT) or cycloheximide (CY; each 10 μg/mL). After incubation, TF activities were determined with a plasma recalcification assay. The means and 1 SD of 8 tissue culture-plate wells are presented.

LPS, with resultant activation of host effector mechanisms. In those studies, mixtures of Hb and LPS were shown to synergistically activate human mononuclear cells and enzymatic coagulation mechanisms.[17] LPS biologic activity in these model systems was clearly shown to be enhanced by Hb. Therefore, modification of LPS by Hb is a process with substantial clinical relevance. In addition, we have recently shown that Hb and LPS form bimolecular complexes and have shown that large LPS aggregates are dissociated on binding to Hb.[17]

Because of the prothrombotic actions of LPS on the vascular endothelium, it was important to investigate the potential ability of Hb to modify this critical interaction during endotoxemia. The present studies showed that HbA_o significantly increased TF activity in HUVEC in response to LPS. This effect was observed at concentrations of Hb (1 to 2 mg/mL) that can be encountered in plasma during clinical and experimental endotoxemia.[14-16] This Hb preparation did not contain detectable erythrocyte stroma that could potentially elicit a HUVEC response. The enhanced TF activity resulted from new TF protein synthesis. The mechanism of this effect is uncertain because the mechanism of LPS signal transduction in HUVEC is not known. However, the demonstration in our laboratory that Hb binds LPS and causes LPS dissociation[17] suggests that disaggregated and/or protein-bound LPS has an increased ability to interact with HUVEC LPS receptors and trigger the procoagulant response. Thus, the Hb enhancement effect may represent the result of presentation to the endothelial cell of a "modified" (eg, disaggregated) LPS. Additionally, a number of other potential mechanisms may be involved. Preliminary studies in our laboratory have shown that LPS induces the denaturation of Hb to methemoglobin and hemichrome, species of Hb that may show altered biologic activity and produce oxygen-free radicals during their formation.

Based on these results using HbA_o, it is reasonable to propose that hemolysis caused by LPS-mediated disseminated intravascular coagulation may constitute a positive feedback loop to amplify coagulation. Additionally, enhancement of other LPS biologic activities by HbA_o, such as mononuclear cell cytokine production, may contribute to the often fatal consequences of low level endotoxemia (during which LPS concentrations are frequently measured in the 10 to 100 pg/mL range[29,30]). The present study also showed enhancement of the TF response to LPS by $\alpha\alpha$Hb, a cross-linked preparation of human Hb that is a leading candidate for use as a blood substitute. These findings raise two concerns for the human use of cell-free Hb. Firstly, contamination of Hb preparations by environmental LPS is difficult to avoid.[19] Because large volumes of Hb would be required for resuscitation, the procoagulant consequences of LPS contamination in the Hb preparations could limit the use of $\alpha\alpha$Hb. In patients receiving 1/10th blood volume of $\alpha\alpha$Hb for resuscitation, blood Hb levels $\geq$10 mg/mL would be achieved. At these concentrations of Hb, substantial enhancement by Hb of the procoagulant activity of any LPS in the circulation would be expected based on our results. Secondly, $\alpha\alpha$Hb would likely be infused into some patients with coexisting endotoxemia. Endotoxemia could arise from gram-negative bacteremia, from reticuloendothelial system dysfunction, and/or from increased gastrointestinal tract translocation of LPS into the circulation secondary to hypotension or trauma. Consequently, $\alpha\alpha$Hb infusion could potentially enhance the systemic pathologic effects of underlying endotoxemia. Because of the demonstration of the in vitro consequences of the interaction between LPS and Hb, which provide a basis for potential thrombotic effects, it is important that future studies investigate the ability of Hb to enhance LPS-induced coagulation in vivo.

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TOXICITY OF HEMOGLOBIN SOLUTIONS: HEMOGLOBIN IS A LIPOPOLYSACCHARIDE (LPS) BINDING PROTEIN WHICH ENHANCES LPS BIOLOGICAL ACTIVITY

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ABSTRACT

Administration of αα-crosslinked stroma-free hemoglobin (SFH) as a cell-free resuscitation fluid is associated with multiple organ toxicities. Many of these toxicities are characteristic of the pathophysiological effects of bacterial endotoxins (lipopolysaccharide, LPS). To better understand the potential role of LPS in the observed in vivo toxicities of SFH, we examined mixtures of SFH and E. coli LPS for evidence of LPS-SFH complex formation. LPS-SFH complexes were demonstrated by three techniques: ultrafiltration through 300 kDa cut-off membranes, which distinguished LPS in complexes (87-89% <300 kDa) from LPS alone (90% >300 kDa); density centrifugation through 5% sucrose, which distinguished denser LPS alone from LPS-SFH complexes; and precipitation by 67% ethanol, which demonstrated 2-3 fold increased precipitability of complexes compared to SFH alone. Interaction of LPS with SFH was also associated with markedly increased biological activity of LPS, as manifested by enhancement of

LPS activation of Limulus amebocyte lysate (LAL), increased release of human mononuclear cell tissue factor, and enhanced production of cultured human endothelial cell tissue factor. These results demonstrated that hemoglobin can serve as an endotoxin binding protein, and that this interaction results in the alteration of several LPS physical characteristics and enhancement of LPS biological activities.

INTRODUCTION

Stroma-free hemoglobin, a preparation of purified human hemoglobin, is being developed for use as a cell-free resuscitation fluid [1-3]. In order to stabilize the protein's tetrameric structure, preparations of stroma-free hemoglobin have been covalently crosslinked between the protein chains. αα-crosslinked stroma-free hemoglobin (SFH) is a modified hemoglobin, crosslinked between the α chains with bis(3,5-dibromosalicyl) fumarate, that demonstrates prolonged in vivo retention. SFH has excellent oxygen binding and delivery properties, as well as an adequate half-life, and therefore is a potentially ideal "blood substitute". However, in vivo administration of SFH has revealed significant problems of toxicity, including hypertension and bradycardia [4,5], a decrease in glomerular filtration rate and renal plasma flow [6], mild prolongations of the partial thromboplastin time [5] and fever. In some studies, administration of SFH has resulted in activation of the complement and coagulation cascades [7-9], disseminated intravascular coagulation with resultant thrombosis [7,10,11], and ischemic parenchymal damage [7,8].

Many of the reported toxicities of SFH infusion can be explained by the known consequences of endotoxemia, and the presence of LPS in preparations of SFH utilized for in vivo studies has been documented [7,9]. Therefore, a contributory role for the observed in vivo toxicity of SFH has been proposed for bacterial endotoxin [10,11]. Previously, SFH and LPS have been shown to produce synergistic in vivo toxicity [11], and we have demonstrated that SFH is capable of enhancing the procoagulant activity of LPS in vitro [12]. We hypothesized that SFH

binds LPS, and that the interaction between these molecules could alter the biological activity of LPS. The present study was designed to determine whether complex formation occurs between SFH and LPS, and evaluate the ability of SFH to alter biologic activities of LPS.

MATERIALS AND METHODS

Reagents. Sterile, 15 ml Falcon tubes were obtained from Becton Dickinson (Mountainview, CA). Sterile, endotoxin-free water and 0.9% NaCl were purchased from Travenol Laboratories (Deerfield, IL).

Glassware. Glassware was heated at 190°C in a dry oven for 4 hours.

Hemoglobin. Human SFH, crosslinked between α chains with bis(3,5-dibromosalicyl)fumarate as described previously [13,14], was provided by collaborators at the Blood Research Division of the Letterman Army Institute of Research (BRD/LAIR), San Francisco, CA. SFH was 9.6 g/dl, pH 7.4, 95.4% crosslinked, 96.3% oxyhemoglobin, 3.2% methemoglobin, and contained less than 0.4 EU/ml endotoxin (referenced to E. coli lipopolysaccharide B, O55:B5, Difco Laboratories, Detroit, MI), as determined by the Limulus amebocyte lysate (LAL) test [15]. The SFH stock solution was stored at -70°C , and then diluted with sterile, pyrogen-free 0.9% NaCl prior to use. Carboxy-SFH(CO-SFH), produced by incubation of the SFH solution with CO, was at 9.6 g/dl, 95.4% crosslinked, 95% HbCO and 5% oxyhemoglobin. Purified non-crosslinked human A_0, 8.4 g/dl, also provided by collaborators at BRD/LAIR, was prepared by ion exchange HPLC of purified human hemoglobin, as described previously [16].

Endotoxin. E. coli O26:B6 lipopolysaccharide (LPS) was obtained from Difco Laboratories (Detroit, MI). ^{14}C-LPS (Salmonella typhimurium PR122(Rc)) was purchased from List Biologicals, Inc. (Campbell, CA) and was resuspended in endotoxin-free water at 1 μCi/ml (1 mg/ml).

Limulus amebocyte lysate (LAL). Amebocyte lysates were prepared from Limulus polyphemus (the North American horseshoe crab) by disruption of washed amebocytes in distilled water [15,17].

Chromogenic Substrate. Chromogenic substrate S-2423 (AB Kabi Vitrum, Molndal, Sweden) was the gift of Dr. Petter Friberger.

Chromogenic Limulus Amebocyte Lysate (LAL) Test. 50 µl of sample and 30 µl of LAL (freshly diluted 1:20 in 0.9% NaCl prior to use) were incubated in tissue culture plates for 30 min at 37°C in a temperature-controlled plate reader (Kinetic-QLC, Whittaker Bioproducts Inc., Walkersville, MD). 40 µl chromogenic substrate S-2423 (0.25 mM, in 25 mM Tris, pH 8.6) was added to each well, mixtures were incubated at 37°C for 5 min, and absorbances at 405 nm then were determined.

Ultrafiltration. Solutions of SFH, CO-SFH or A_0 were prefiltered through an endotoxin-free 300 kDa membrane prior to use to remove aggregated protein particles. SFH, CO-SFH or A_0 (100 µg/ml) was incubated with E. coli O26:B6 (W) LPS (50 µg/ml) for 30 min at 37°C. Mixtures then were filtered manually with a 3 ml syringe (according to the directions of the filter manufacturer) at room temperature, using a 300 kDa cut-off filter (ultrafree-PFL polysulfone 300, Millipore Corporation, Bedford, MA). LPS concentrations in filtered solutions of hemoglobin, hemoglobin and LPS mixtures, or LPS alone were determined by the chromogenic LAL test (described above), using starting mixtures of hemoglobin-LPS, or LPS alone, for the standard curve. Hemoglobin protein concentrations were determined by the BCA protein assay (Pierce, Rockford, IL).

Sucrose centrifugation of LPS and SFH. ^{14}C-S. typhimurium LPS (0.005 µCi) was added to each of the hemoglobin solutions (each diluted to 10 mg/ml), and the mixtures were incubated for 30 min at 20°C. Aliquots of LPS-hemoglobin mixtures, LPS alone, or hemoglobin alone then were layered over 5% pyrogen-free sucrose and centrifuged at 2,900 x g for 30 min at 20°C, in a Sorvall RC-5 centrifuge (Du Pont Instruments, Wilmington, DE). Scintillation counting was performed, after samples were diluted 10-fold in fluor (Formula A-989, NEN Research Products, Boston, MA), in a Tracor Analytic Liquid Scintillation System (Tracor Analytic, Elk Grove Village, IL). For samples containing hemoglobin, quenching of ^{14}C-LPS by hemoglobin was reversed as follows: 0.1 ml aliquots of fractions were diluted ten-fold in water (to 1 ml final volume), and 1 ml Solvable (NEN Research Products, Boston, MA) was added. These mixtures were incubated at 60°C for one hr, and then 0.3 ml 25% H_2O_2 was added.

Ethanol precipitation of SFH and LPS-SFH mixtures. 2 μg SFH, CO-SFH, or A_0 was incubated with 25 μg E. coli O26:B6 (W) LPS in microtiter plate wells for 30 min at 20°C. Ethanol then was added to each well (final concentration, 67%), and after an additional 30 min the mixtures were centrifuged at 800 x g for 30 min. The concentrations of hemoglobin in the sediments were determined by protein assay, and LPS concentrations by the phenol-concentrated H_2SO_4 method [18].

Mononuclear cell (MNC) tissue factor (TF) assay. E. coli LPS (100 ng/ml) was incubated with SFH (6 mg/ml) for 30 min at 37°C. LPS alone, SFH alone, or LPS-SFH mixtures were then incubated for 20 hr at 37°C with human peripheral blood MNC [19] and assayed for TF with a one-stage coagulation assay [20]. A clotting time of 30 sec was defined as equal to 100 units TF activity [21].

Endothelial cell tissue factor (TF) assay. Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics (San Diego, CA) and cultured in media (containing 2% serum) obtained from Clonetics. Cells were grown to confluent monolayers in sterile 96-well microtiter plate wells (Nunclon, Applied Scientific, San Francisco). E. coli LPS alone, SFH alone, or LPS-SFH mixtures were added to the media in each well (final concentrations: 1 μg/ml LPS; 1 mg/ml SFH), and incubated for 4 hr. Wells were then washed with media (x 3) and the HUVEC were freeze-thawed (x 2) and sonicated in phosphate buffered saline. To each well then was added normal human citrated plasma and calcium (25 mM), and plates were incubated for 8 min in a temperature-controlled (37°C) plate reader (Kinetic-QLC, Whittaker Bioproducts Inc., Walkersville, MD). Turbidity was measured at 340 nm, and TF activity was calculated from a standard curve established with rabbit brain thromboplastin (Baxter Corporation, Miami, FL). The turbidity generated at 8 min by 1:100 diluted thromboplastin was arbitrarily defined as 10 TF units.

RESULTS

Demonstration of LPS-SFH complexes. Ultrafiltration experiments demonstrated that 87-89% of the LPS in LPS-SFH mixtures was

filtered through the 300 kDa membrane, whereas only 10% of LPS alone was filterable (Fig. 1). This indicated that SFH caused the dissociation of LPS into lower molecular weight particles. Approximately 90% of the total SFH protein in each of the three LPS-SFH mixtures, and from filtrates of SFH alone, was detected in filtrates (data not shown). Utilizing ethanol precipitation, greater than twice the amount of each SFH was precipitated in the presence of LPS than was with SFH alone (Fig. 2). In both the absence and presence of SFH, approximately 90% of LPS was precipitated by ethanol (data not shown). Following sucrose centrifugation, 76% of LPS sedimented into the bottom fraction in the absence of protein, whereas only 3-9% sedimented in the presence of any of the three SFH preparations (Figure 3). Conversely, only 3% of LPS alone remained above the sucrose layer, whereas in the presence of SFH, 64-79% of LPS remained in the top layer. No detectable SFH entered the sucrose layer in either the absence or presence of LPS. Therefore, SFH decreased the density of LPS, resulting in the co-migration of SFH and LPS.

Biological activity of LPS in SFH-LPS complexes. SFH increased the biological activity of LPS in three independent assays. First, LPS in the presence of SFH produced enhanced activation of LAL (3 to 4.5-fold) compared to LAL activation by LPS alone (Fig. 4). Second, LPS-SFH complexes resulted in 5.5-fold greater TF production by human MNC than the TF generated from MNC by LPS alone (Fig. 5). Third, SFH resulted in a 2.8-fold increase in endothelial cell TF production compared to TF generated by LPS alone (Fig. 6).

DISCUSSION

We performed experiments to determine whether SEH interacted with LPS. Ultrafiltration demonstrated that the molecular weight of LPS (typically $>10^6$ in aqueous solution) was reduced to < 300 kDa in the presence of SFH, and that LPS and SFH co-filtered. Utilizing centrifugation through sucrose, we showed that the density of LPS in the presence of SFH was distinctly less than that of LPS alone, and that LPS and SFH co-migrated. Measurement of SFH precipitation by

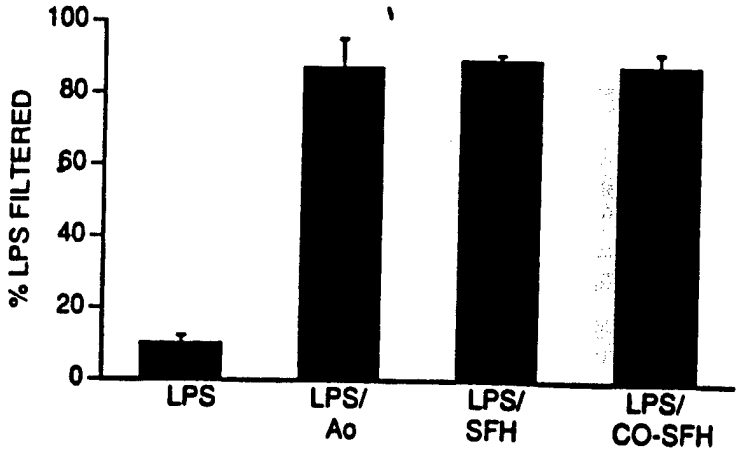

FIGURE 1. Ultrafiltration of SFH and LPS. E. coli LPS was incubated with SFH, CO-SFH or A_0, and the mixtures were filtered through a 300 kDa cut-off ultrafiltration membrane. The % of LPS filtered was deter-mined by the LAL test. All three preparations of SFH greatly increased the filterability of LPS.

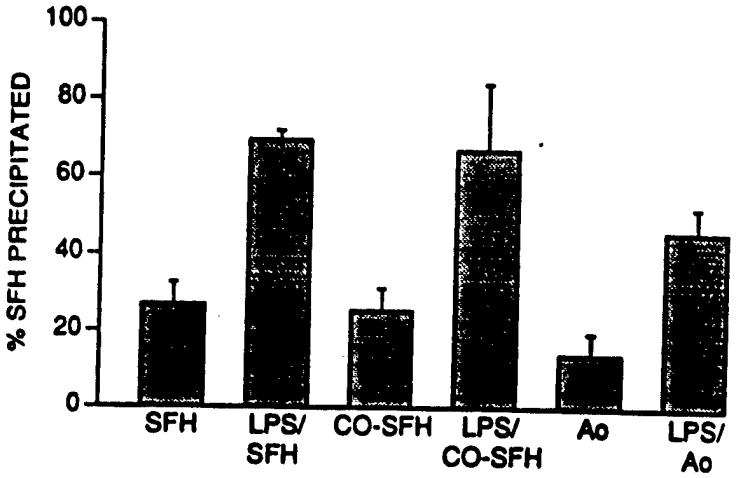

FIGURE 2. Precipitation of SFH and LPS by ethanol. E. coli LPS was incubated with SFH, CO-SFH, or A_0, and the LPS-SFH complexes were then precipitat-ed from the mixtures by 67% ethanol and sedimented by cen-trifugation. Each preparation of SFH demonstrated increased precipitability in the presence of LPS.

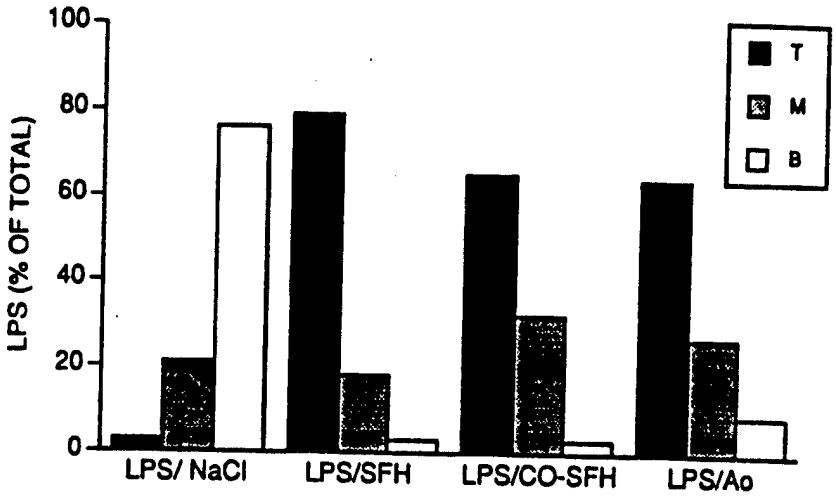

FIGURE 3. Centrifugation of SFH and LPS through sucrose. ^{14}C S. typhimurium LPS was incubated with SFH, CO-SFH, or A_o, and the mixtures were then centrifuged through 5% sucrose. The distributions of radiolabeled LPS were determined in top (T), middle (M) and bottom (B) zones of the centrifuged samples. All three preparations of SFH co-migrated with LPS, resulting in a decrease in LPS density.

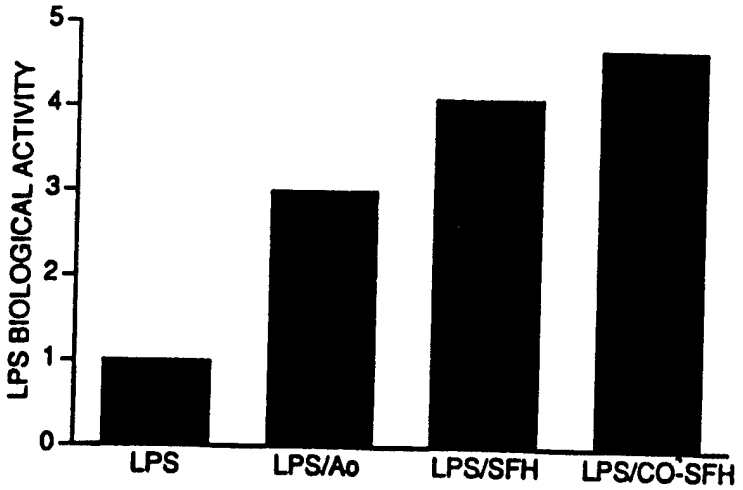

FIGURE 4. Effect of SFH on LPS biological activity in the LAL test. E. coli LPS, in the absence or presence of SFH, CO-SFH, or A_o was incubated with LAL, and activation measured with a chromogenic substrate. LPS biological activities in LPS-protein mixtures are expressed as relative activities to LPS alone. All three preparations of SFH resulted in increased biological activity of LPS.

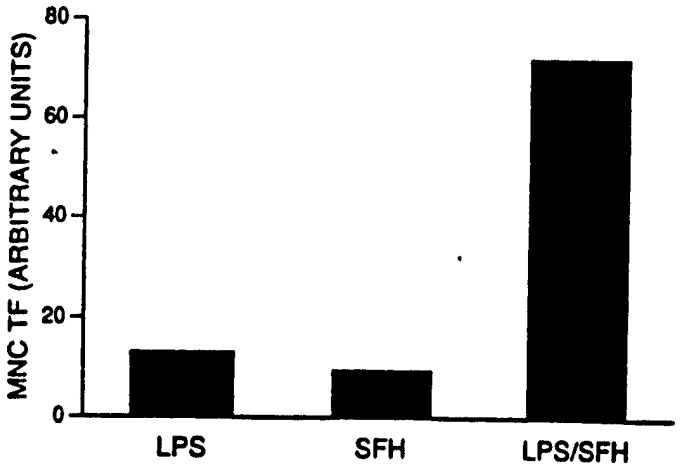

FIGURE 5. Effect of SFH on the LPS-induced stimulation of TF activity in human MNC. E. coli LPS, in the absence or presence of SFH, was incubated with human MNC, and TF activity was measured with a plasma clotting assay. SFH enhanced the ability of LPS to stimulate TF production.

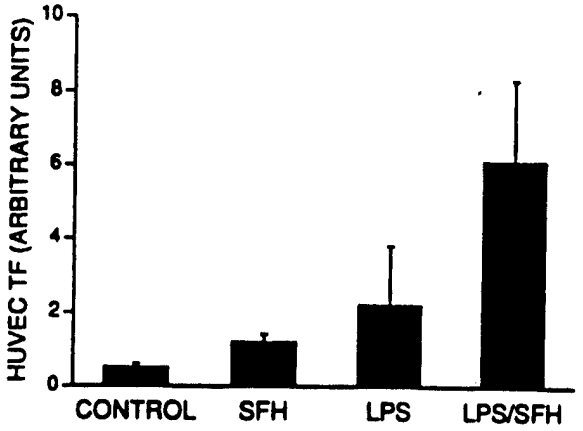

FIGURE 6. Effect of SFH on the LPS-induced stimulation of TF activity in HUVEC. E. coli LPS, in the absence or presence of SFH, was incubated with HUVEC, and TF activity was measured with a plasma clotting assay. SFH enhanced the LPS-induced production of TF from the endothelial cells.

ethanol indicated that LPS greatly increased the precipitability of SFH. Therefore, our experiments demonstrated that the physical characteristics of both SFH and LPS were altered in LPS-SFH mixtures. These results are consistent with the formation of stable complexes, and establish the ability of hemoglobin to act as an endotoxin-binding protein. Because these results were observed with unmodified hemoglobin (A_0) and CO-SFH (which was not susceptible to methemoglobin production), as well as with SFH, we have demonstrated that LPS-binding is an intrinsic property of hemoglobin.

The formation of LPS-SFH complexes was associated with major changes in the procoagulant activities of LPS. SFH enhanced the ability of LPS to stimulate coagulation via three independent mechanisms: 1) direct activation of the proteolytic coagulation cascade of Limulus, 2) stimulation of TF production from human MNC, and 3) stimulation of TF production from HUVEC. Enhancement by SFH of LPS procoagulant activity may contribute to the observed thrombosis and ischemic damage associated with SFH infusion in animals [7,8], and may also provide a mechanism for the synergistic toxicity between SFH and LPS reported previously [11,22]. Interestingly, other proteins that are known to bind LPS with a resultant change in LPS biological activity (e.g., mellitin [23], lysozyme [24], and complement [25] or the polypeptide polymyxin B [26]) cause a decrease in LPS toxicity.

Our observations that LPS, when complexed with SFH, was of much lower molecular weight and lesser density than LPS alone suggest that SFH caused the disaggregation of LPS. In contrast to the increased biological activity we observed for LPS that had been disaggregated and bound to SFH, the process of LPS disaggregation in plasma (resulting primarily from its interaction with high density lipoproteins [27]), results in detoxification. It is possible that the process of LPS-SFH complex formation might potentially interfere with LPS detoxification in plasma.

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conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army. This work also was supported, in part, by Research Grant DK 43102 from the NIDDKD, National Institutes of Health; and the Veterans Administration.

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The effect of cell-free hemoglobin on intravascular clearance and cellular, plasma, and organ distribution of bacterial endotoxin in rabbits

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Cell-free hemoglobin (Hb) and bacterial endotoxin (LPS) synergistically produce toxicity. To elucidate possible mechanisms, three groups of rabbits received LPS alone, LPS plus human serum albumin (HSA), or LPS plus Hb (Hb group). The intravascular retention of injected iodine 125–labeled LPS during the 30-minute period analyzed was significantly longer in the Hb group than in the LPS alone or HSA groups ($p = 0.0007$ and $p = 0.03$, respectively), especially during the initial 10 minutes. The intravascular half-life of LPS in the LPS control, HSA, and Hb groups was 2.8, 4.0, and 4.9 minutes, respectively; the area under the curve was 1369 ± 483, 1594 ± 360, and 1731 ± 481, respectively (ng/ml × minutes, mean ± SD); and the total body clearance was 24.7 ± 9.2, 20.1 ± 5.4, and 18.9 ± 6.0 (ml/min, mean ± SD), respectively. The proportion of LPS associated with blood cells was very small at the initial 1-minute time period, and this decreased even further during the 30-minute period analyzed ($p = 0.0001$). Over 96% of injected LPS was associated with the cell-free plasma, with 51% to 54% of LPS in the apoprotein fraction at the initial time point and 35% to 37% in the HDL fraction. The proportion of LPS increased significantly in the HDL fraction and decreased significantly in apoproteins during the 30-minute period analyzed ($p = 0.0006$ and $p = 0.002$, respectively). However, there were no differences between the three groups. The liver was the main distribution site (74%) of injected LPS among the six organs evaluated. In the Hb group the accumulation of ^{125}I-labeled LPS in the spleen was significantly lower than that in the HSA group ($p = 0.05$). The synergism of the in vivo toxicity reported for LPS and Hb may be due, in part, to the decreased rate of intravascular clearance of endotoxin. (J Lab Clin Med 1995;126:151-60).

Abbreviations: AUC = area under the curve; EDTA = ethylenediaminetetraacetic acid; Hb = cell-free hemoglobin; HDL = high-density lipoprotein; HSA = human serum albumin; LDL = low-density lipoprotein; LPS = lipopolysaccharide, bacterial endotoxin; MNC = mononuclear cell; PBS = phosphate-buffered saline solution; PMN = polymorphonuclear leukocyte; VLDL = very-low-density lipoprotein; WBC = white blood cell

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Opinions, interpretations, conclusions, and recommendations ar those of the authors and are not necessarily endorsed by the U.S Army.

In conducting research using animals, the investigators adhered t the Guide for the Care and Use of Laboratory Animals prepared b the Committee on Care and Use of Laboratory Animals of the Inst tute of Laboratory Animal Resources, National Research Counc (NIH publication no. 86-23, revised 1985).

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Cell-free hemoglobin, being developed as an erythrocyte substitute,[1-3] has demonstrated toxicity in some human trials.[4,5] The mechanisms are unclear, but such toxicity could theoretically result from Hb itself, from residual contaminants such as erythrocyte stroma, or from LPS.[6,7] LPS is the cell wall component of gram-negative bacteria responsible for fever, cardiovascular shock, and disseminated intravascular coagulation during sepsis.[8] When Hb solutions are used as a resuscitation fluid after trauma or shock, endotoxin is likely to be in the circulation. In addition, the infused cell-free Hb may have been contaminated by environmental endotoxin during its production.[9] Synergistic in vivo toxicities from endotoxin and Hb have been reported, including an increased frequency of ventricular arrythmias, prolongation of the thrombin time, and increased mortality.[10] In addition, it has recently been reported that in vitro, endotoxin and Hb form complexes, and these complexes have increased potency for the production of tissue factor by endothelial cells[11] and monocytes,[9] and the activation of coagulation proteases.[12] However, the mechanism(s) of their synergistic interaction has not been fully explored.

One plausible mechanism for synergistic in vivo toxicity is that Hb potentiates the deleterious host pathophysiologic responses to LPS by prolonging the intravascular retention time or by altering cellular, plasma, or organ distribution of LPS. When LPS is administered parenterally to animals, much of the injected LPS is initially found in the cell-free fraction of plasma, with special affinity for HDL.[13,14] HDL-bound LPS has reduced biologic activities.[13] Recently it has been demonstrated that LDL,[15] VLDL,[16] and chylomicrons[16] also detoxify LPS. It is possible that Hb may change the pattern of LPS binding to these lipoproteins and also to other plasma proteins, such as LPS binding protein[17] or soluble CD14,[18] that are presently believed to be critical for the binding of LPS to cellular receptors and the subsequent process of signal transduction. Interactions between Hb-LPS complexes and circulating blood cells are of particular interest, because during sepsis, many of the deleterious effects of LPS are the result of mediators released from inflammatory cells.[8]

Recently we have described the in vitro distribution of LPS between cells, lipoproteins, and plasma proteins in the presence and absence of Hb.[19] Hb has been shown not to appreciably alter the distribution of LPS when LPS is added to blood in vitro. However, it is possible that such an effect occurs in vivo. Furthermore, the potential alteration by Hb of the blood and organ distribution of LPS in vivo has not been examined but is of clinical interest, because there are many reports of alterations of peripheral blood cell levels after administration of LPS in animal models[20-22] and to human volunteers[23,24] and in human gram-negative sepsis.[25] The current study was undertaken to elucidate potential mechanisms for synergistic toxicity produced by LPS and Hb related to possible alterations in intravascular clearance or in cellular and plasma distribution of LPS.

METHODS

Reagents and labware. Percoll and Ficoll were purchased from Pharmacia LKB, Alameda, Calif. EDTA blood tubes and Falcon centrifuge tubes were obtained from Becton Dickinson, Mountain View, Calif. Capillary tubes were from Drummond Scientific Co., Broomall, Pa. Sterile PBS was from GIBCO Laboratories, Grand Island, N.Y.

LPS. *Escherichia coli* O26:B6 LPS (Difco Laboratories, Detroit, Mich.) was purified and the sodium salt was produced by electrodialysis, as described previously.[26] Radioiodination of the purified LPS with ^{125}I was then performed.[27] The specific activity was 1.1 μCi/μg. The iodinated LPS was examined by gel permeation chromatography to ensure that the radiolabel was located on chemically intact LPS molecules. ^{125}I-labeled LPS in water and ^{125}I-labeled LPS in plasma (obtained 20 to 30 minutes after intravenous injection into rabbits, as described below in the experimental protocol section) were each chromatographed on a Superose 12 column with an FPLC system (Pharmacia LKB, Uppsala, Sweden). Seventy-three percent of ^{125}I-labeled LPS in water eluted in the void volume ($>2 \times 10^6$ daltons), and the remainder was broadly distributed between approximately 10^4 daltons (consistent with estimates of the approximate molecular weight of monomeric LPS) and 10^6 daltons. ^{125}I-labeled LPS in plasma demonstrated 29% of the radioactive material in the void volume, with the remainder distributed within the included volume (10^4 to 10^6 daltons). There was no free ^{125}I and no new peak of very low molecular weight material ($<10^4$ daltons) that would be present if monomeric LPS had been enzymatically degraded in vivo.

Hb. Human αα cross-linked hemoglobin, prepared as described previously,[28] was provided by collaborators at the Blood Research Division of the Letterman Army Institute of Research, San Francisco. The Hb solution used in these experiments was at a concentration of 10 gm/dl and contained less than 10 pg/ml endotoxin, as determined by the *Limulus* amebocyte lysate test.[29,30]

Experimental protocol. New Zealand White female rabbits (1.6 to 3.4 kg; Western Oregon Rabbit Co., Philomath, Ore.) were divided into three groups: (1) LPS only (n = 6), (2) LPS plus HSA (n = 6), and (3) LPS plus Hb (n = 5). To study the distribution of ^{125}I-labeled LPS in whole blood, in vivo, a bolus injection of 13 μg/kg ^{125}I-labeled LPS in 0.5 ml of distilled water was given intravenously into a marginal ear vein in all groups. The latter two groups of rabbits were infused intravenously with either endotoxin-free HSA (10 gm/dl) (Travenol Labs, Hyland Therapeutics Division, Glendale, Calif.) or Hb (10 gm/dl) over a 10-minute period just before the injection of ^{125}I-labeled LPS.

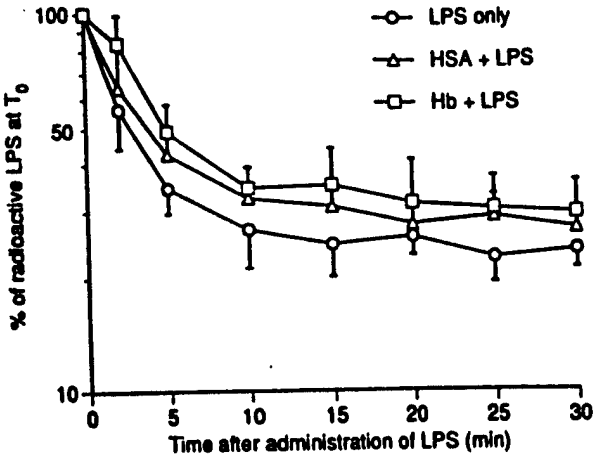

Fig. 1. Intravascular clearance of ^{125}I-labeled LPS after intravenous injection into rabbits. ○, LPS only; △, LPS administered immediately after a 10-minute infusion of HSA; □, LPS administered after Hb. The numbers of animals in each group were 6, 6, and 5, respectively. Values represent the mean percent of the level of radioactive LPS in whole blood at T_0, ± SD.

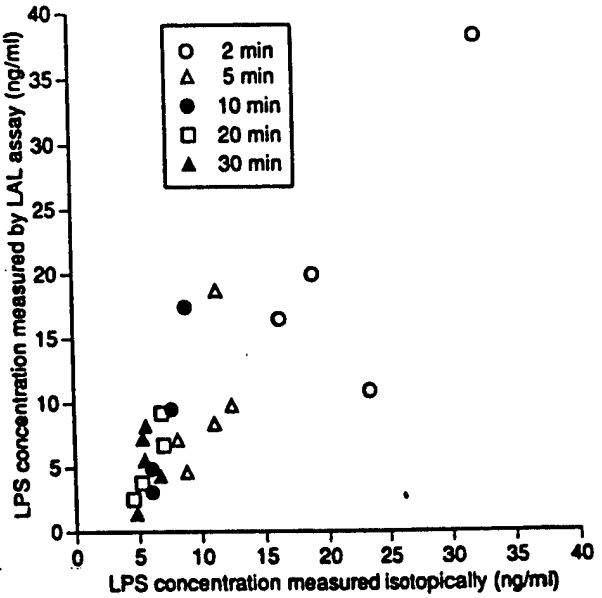

Fig. 2. Comparison of LPS concentrations in the circulation, measured isotopically (^{125}I) and biologically (by the *Limulus* test). ○, 2 minutes; △, 5 minutes; ●, 10 minutes; □, 20 minutes; and ▲, 30 minutes after intravenous administration of LPS.

The volume of HSA or Hb infused was equal to 25% of their blood volume (estimated at 70 ml/kg). No distress was produced by these infusions. Blood samples for measurement of the intravascular clearance of LPS were drawn from the opposite ear vein by using a heparinized capillary tube (70 μl) at 0.25, 0.5, 1, 2, 3, 5, 10, 15, 20, 25, and 30 minutes after injection of ^{125}I-labeled LPS. Radioactivity of the whole blood samples was measured with a Packard model 500 gamma counter (Packard Instrument Co., Downers Grove, Ill.).

Blood samples (3 ml) for blood cell counts and for the fractionation of cellular and plasma components were also drawn at 1, 5, 10, 20, and 30 minutes after injection of ^{125}I-labeled LPS. Blood samples for blood cell counts also were obtained from another 2 rabbits after injection of 0.5 ml of distilled water.

To study the distribution of ^{125}I-labeled LPS in whole blood in vitro, blood samples (3 ml) were collected from all rabbits before infusion of Hb or HSA and 3 minutes after protein infusions. These samples then were incubated with 200 μl of radiolabeled LPS (100 ng) for 20 minutes at room temperature.

Hb determination. Free hemoglobin in plasma, in the rabbits that received Hb, was determined by using an H1 particle counter (Technicon Instrument Corp., Tarrytown, N.Y.). Hb infusion was associated with a 2 gm/dl increase in blood hemoglobin level (from 12.0 gm/dl to 14.0 gm/dl).

Analysis of intravascular ^{125}I-labeled LPS clearance. To determine the intravascular LPS clearance, the T_0 value (cpm/ml whole blood) was calculated by using the following formula: total cpm of injected ^{125}I-labeled LPS/estimated blood volume (70 ml/kg × rabbit weight [kg] + volume of protein solution [ml]). Intravascular half-life was determined from the clearance curve. The area under the concentration-time curve (AUC in units of ng/ml × minutes) was calculated by trapezoid integration, $\Sigma[\frac{1}{2}(t_{n+1} - t_n)(C_{n+1} + C_n)]$.[31] The early data points (0.25 and 0.5 minutes) were not used for the calculation because of insufficient mixing of injected LPS with the entire blood volume. Total body clearance (in units of ml/min) = total dose of injected ^{125}I-labeled LPS/AUC.

Plasma LPS concentration determined by the endotoxin-specific chromogenic *Limulus* test. To compare the clearance of LPS from blood, as determined by both isotopic measurement and biologic activity, 1 ml samples of whole blood were also drawn at 1, 5, 10, 20, and 30 minutes after injection of ^{125}I-labeled LPS into sterile EDTA tubes from 5 animals (1 of the LPS-only group, 2 of the HSA group, and 2 of the Hb group) to determine the plasma LPS levels by using an endotoxin-specific chromogenic *Limulus* test (Endospecy; Seikagaku Corp., Tokyo, Japan).[32,33] This test consists of the clotting enzymes of the amebocyte lysate from the Japanese horseshoe crab *Tachypleus tridentatus* and a chromogenic substrate, butoxycarbonyl-leu-gly-arg-*p*-nitroanilide. *Escherichia coli* 0111:B4 endotoxin (Difco Laboratories) was used for the standard.

Leukocyte and platelet counts. Leukocyte and platelet counts were determined with blood cell counters (Coulter Electronics Inc., Hialeah, Fla.). Leukocyte differentials were determined by 250-cell manual counts of Wright-Giemsa-stained smears.

Fractionation of whole blood. Fractionations were performed at room temperature as described previously.[19] In brief, 3 ml samples, diluted with 6 ml of PBS-0.15 EDTA, were centrifuged in an Accuspin centrifuge (Beckman Instruments Inc., Irvine, Calif.) at 600 *g* for 3 minutes to obtain platelet-rich plasma and a cell pellet. A 5 ml sample of PBS-EDTA then was added to the cell pellet which was resuspended and recentrifuged at 600 *g*. The

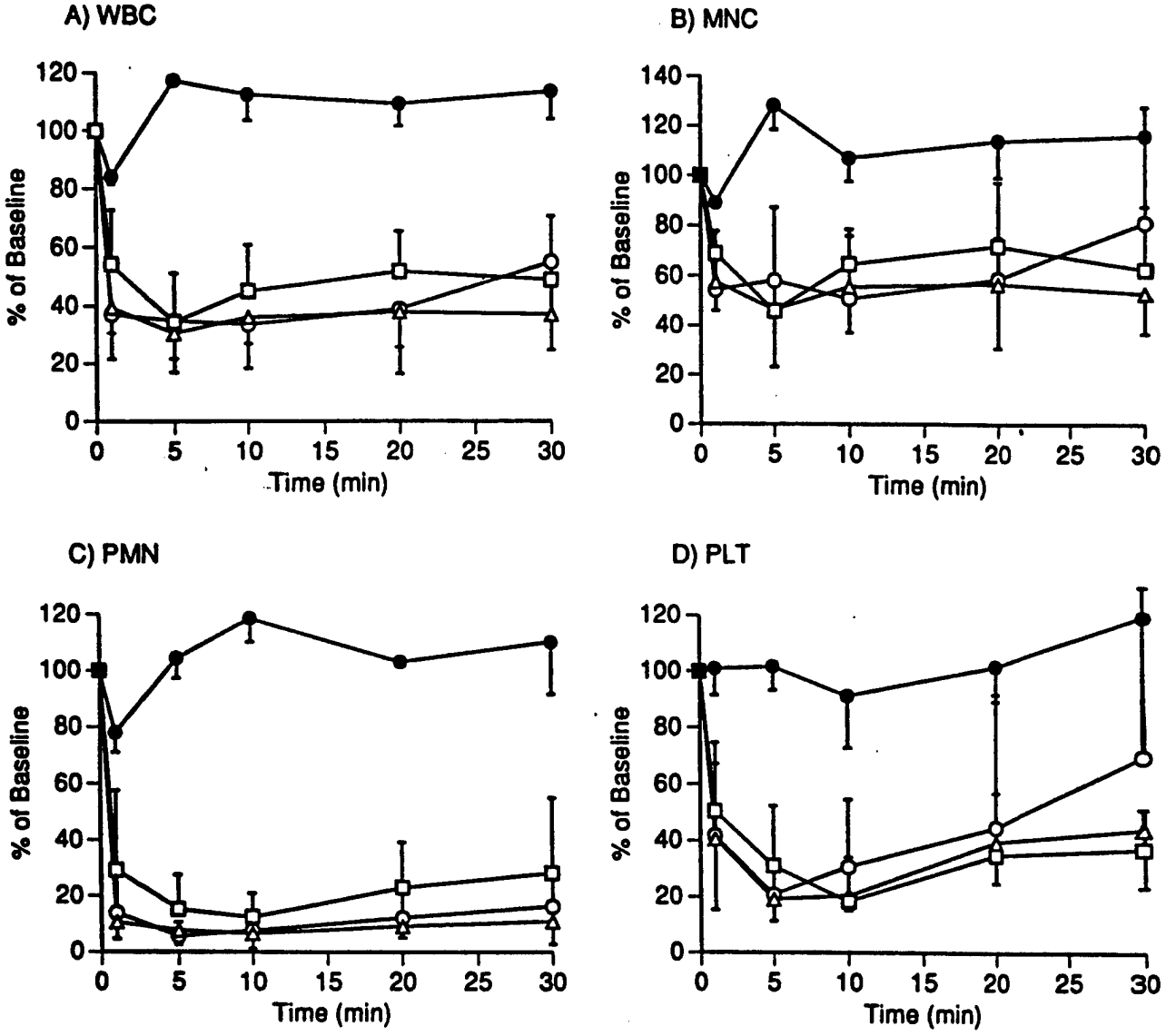

Fig. 3. The effect of ^{125}I-labeled LPS injection on (A) total WBCs, (B) MNCs, (C) PMNs, and (D) platelets (PLT). ○, LPS only; △, HSA plus LPS; □, Hb plus LPS. The numbers of animals in each group were 6, 6, and 5, respectively. The lack of effect of injection of 0.5 ml distilled water (in which LPS was administered) on blood cell counts also is shown (●, n = 2). Values represent mean percent of the baseline counts ± SD. The baseline levels of total WBCs, MNCs, PMNs, and platelets were 6663 ± 2297/μl, 4266 ± 1804/μl, 2398 ± 994/μl, and 380,833 ± 148,213/μl (n = 17), respectively.

sultant supernatant then was combined with the initial platelet-rich plasma. The combined platelet-rich plasma was then centrifuged at 1300 *g* for 25 minutes to obtain a platelet pellet and cell-free plasma. The platelet pellet was washed twice with PBS-EDTA at 250 *g* for 10 minutes. The cell pellet from the initial whole-blood centrifugation (leukocytes and erythrocytes) was resuspended in PBS-EDTA, layered over Ficoll (density = 1.070 gm/ml), and centrifuged at 400 *g* for 40 minutes. MNCs were collected at the interface and washed once with PBS-EDTA for 10 minutes (250 *g*). The Ficoll-pelleted cells (PMNs and erythrocytes), resuspended in PBS-EDTA, were layered onto Percoll (density = 1.070 gm/ml) and centrifuged at 400 *g* for 20 minutes. PMNs present at the plasma-Percoll interface and erythrocytes in the pellet were collected.

Separation of lipoproteins from cell-free plasma. The cell-free plasma was subjected to sequential ultracentrifugation at 8° C at plasma density (density = 1.006 gm/ml) for 18 hours, then at a density of 1.063 gm/ml with KBr for 18 hours, and finally at a density of 1.21 gm/ml with KBr for 40 hours for isolation of VLDL, LDL, and HDL, respectively, and for isolation of apoproteins (i.e., plasma proteins remaining after sequential removal of all lipoproteins; density > 1.21 gm/ml).[19,34]

Distribution of ^{125}I-labeled LPS in tissues. Distribution of ^{125}I-labeled LPS in tissues was studied in animals killed 40

Table I. Endotoxin distribution in whole blood

	% Plasma			% Cells		
	Control	HSA	Hb	Control	HSA	Hb
Time after injection of LPS						
1 min	96.0 ± 1.4	97.0 ± 1.4	96.4 ± 1.4	4.0 ± 1.4	3.0 ± 1.4	3.6 ± 1.4
5 min	97.8 ± 0.2	97.7 ± 0.9	98.0 ± 0.3	2.2 ± 0.2	2.4 ± 0.9	2.0 ± 0.3
10 min	97.9 ± 0.6	98.1 ± 0.9	98.4 ± 0.1	2.1 ± 0.6	1.9 ± 0.9	1.6 ± 0.1
20 min	98.0 ± 0.7	98.4 ± 1.1	98.3 ± 0.2	2.0 ± 0.7	1.6 ± 1.1	1.7 ± 0.2
30 min	97.6 ± 1.2	98.6 ± 1.1	98.2 ± 0.2	2.4 ± 1.2	1.4 ± 1.1	1.8 ± 0.2
In vitro incubation						
Before protein infusion	96.4 ± 0.9	95.7 ± 1.7	97.2 ± 0.7	3.6 ± 0.9	4.3 ± 1.7	2.8 ± 0.7
After protein infusion	ND	96.8 ± 0.6	96.9 ± 1.2	ND	3.2 ± 0.6	3.1 ± 1.2

^{125}I-labeled *E. coli* O26:B6 LPS was injected into rabbits after infusion of hemoglobin or HSA solutions or added to whole blood in vitro. Then blood samples were fractionated into cell-free plasma, platelets, MNCs, PMNs, and erythrocytes (RBCs). *Percent cells* represents the total of the four cellular fractions. Endotoxin distributions are expressed as mean ± SD.

minutes after ^{125}I-labeled LPS injection. Liver, spleen, kidney, adrenal, lung, and heart were weighed, and small samples (also weighed) were taken for measurement of radioactivity.

Data analysis. A two-tailed *t* test and analysis of variance with repeated measures were performed. A p value less than 0.05 was deemed significant. All data are presented as the mean ± SD except as otherwise noted.

RESULTS

Intravascular clearance of ^{125}I-labeled LPS. ^{125}I-labeled LPS clearance was measured in rabbits, in the presence and absence of cell-free hemoglobin, to determine whether there was a physiologic interaction between the two substances. Three groups were analyzed: (1) ^{125}I-labeled LPS alone (control group), (2) ^{125}I-labeled LPS in the presence of preinfused HSA (HSA group), and (3) ^{125}I-labeled LPS in the presence of preinfused αα cross-linked hemoglobin (Hb group). Fig. 1 shows the pattern of LPS intravascular clearance during the 30-minute period after administration of ^{125}I-labeled LPS. There was a significant difference between the Hb and control group or HSA group ($p = 0.0007$ and $p = 0.03$, respectively, analysis of variance) when the entire 30-minute curves were analyzed. The difference was maximum during the first phase of clearance (initial 10-minute period; $p = 0.0003$, Hb vs control, and $p = 0.03$, Hb vs HSA) when the decay slopes were visibly different but was less so during the second phase of clearance (10-to-30-minute period; $p = 0.06$, Hb vs control, and $p = 0.4$, Hb vs HSA). Comparison of counts per minute at the early time points of individual animals in the Hb and control (LPS only) groups demonstrated that there was no overlap between the groups at 1 minute and that 4 of 5 values in the Hb group were outside of the range of levels in the LPS-only group, both at 2 and 5 minutes. Thirty minutes after the bolus injection of ^{125}I-labeled LPS, the remaining counts per minute of whole blood for the control, HSA, and Hb groups were 23.6% ± 2.5%, 26.9% ± 3.3%, and 29.4% ± 6.3% of the calculated T_0 values, respectively. There were mean values of 14% and 25% more LPS remaining in the blood of the Hb group than in the HSA and control groups, respectively, at 30 minutes. The intravascular half-life of LPS in the control, HSA, and Hb groups was 2.8, 4.0, and 4.9 minutes, respectively (not significantly different). The area under the concentration-time curve of the control, HSA, and Hb groups was 1369 ± 483, 1594 ± 360, and 1731 ± 481 (ng/ml × min), respectively. The total body clearance in the three groups was 24.7 ± 9.2, 20.1 ± 5.4, and 18.9 ± 6.0 (ml/min), respectively (not significantly different).

To demonstrate that the isotopically determined LPS concentrations in blood represented biologically active material, plasma LPS concentrations in 5 animals also were determined with the *Limulus* test. Similar to the isotopic clearance, there was rapid clearance of biologically active LPS from the circulation (data not shown). The relative concentrations of LPS, as determined isotopically and by the *Limulus* test, were similar at each of the time points (Fig. 2). This indicated that the radiolabeled LPS was representative of the biologically active material in the circulation.

Blood cell counts. The alterations of blood cell counts after injection of ^{125}I-labeled LPS are shown in Fig. 3. A rapid decrease in WBC levels, especially of PMNs, along with severe thrombocytopenia was observed maximally within 5 minutes. There were no statistically significant differences between the three groups. Hematocrit levels decreased slightly, consistent with the volume of blood removed for samples (data not shown).

Distribution of ^{125}I-labeled LPS in blood. The distribution of ^{125}I-labeled LPS was determined after injection

Table II. Endotoxin distribution in blood cellular compartment

	% PLTs			% MNCs		
	Control	HSA	Hb	Control	HSA	Hb
Time after injection						
1 min	43.9 ± 3.8	28.1 ± 14.0	38.9 ± 22.3	5.0 ± 3.7	1.9 ± 2.4	6.3 ± 5.6
5 min	23.8 ± 5.2	21.9 ± 17.4	12.9 ± 9.8	6.5 ± 7.2	0.7 ± 1.1	2.2 ± 3.3
10 min	22.9 ± 18.7	19.4 ± 22.1	9.7 ± 3.9	4.4 ± 4.0	1.0 ± 2.1	0 ± 0
20 min	26.5 ± 22.3	10.4 ± 14.9	15.4 ± 4.2	2.6 ± 2.8	1.6 ± 3.0	3.3 ± 1.7
30 min	29.8 ± 39.1	13.5 ± 20.1	21.0 ± 4.5	4.0 ± 5.6	1.5 ± 1.7	2.8 ± 4.9
In vitro incubation						
Before protein infusion	22.3 ± 11.0	41.2 ± 23.4	43.3 ± 14.1	6.0 ± 5.7	3.3 ± 6.2	0.5 ± 0.4
After protein infusion	ND	51.2 ± 4.0	52.1 ± 3.1	ND	4.7 ± 4.8	3.2 ± 4.8

^{125}I-labeled *E. coli* O26:B6 LPS was injected into rabbits after infusion of hemoglobin or HSA solutions or added to whole blood in vitro. Then blood samples were fractionated into cell-free plasma, platelets (PLTs), MNCs, PMNs, and erythrocytes (RBCs). Endotoxin distributions are expressed as mean ± SD. 100% = total cpm LPS in the cellular compartment.

Table III. Endotoxin distribution in cell-free plasma

	Endotoxin distribution (% total cpm in plasma)					
	VLDL			LDL		
	Control	HSA	Hb	Control	HSA	Hb
Time after injection						
1 min	6.1 ± 0.3	6.1 ± 0.4	6.4 ± 0.6	6.6 ± 2.1	4.7 ± 0.5	6.0 ± 0.8
5 min	7.0 ± 0.1	6.8 ± 0.6	7.0 ± 0.9	6.8 ± 1.7	5.0 ± 0.7	6.5 ± 1.0
10 min	7.0 ± 0.3	7.0 ± 0.5	6.6 ± 0.5	6.8 ± 1.7	5.4 ± 0.2	6.7 ± 1.0
20 min	6.9 ± 0.9	5.9 ± 0.2	6.4 ± 0.4	6.9 ± 2.1	5.1 ± 0.5	6.5 ± 0.5
30 min	7.0 ± 0.8	6.2 ± 0.1	6.5 ± 0.2	7.1 ± 1.8	5.2 ± 0.7	7.2 ± 1.1
In vitro incubation						
Before protein injection	6.7 ± 0.7	6.3 ± 0.3	6.1 ± 0.5	6.8 ± 2.1	5.1 ± 0.2	6.3 ± 1.1
After protein injection	ND	6.0 ± 0.3	6.3 ± 0.8	ND	4.8 ± 0.1	6.1 ± 1.5

^{125}I-labeled *E. coli* O26:B6 LPS was injected into rabbits after infusion of hemoglobin or HSA solutions or added to whole blood in vitro. Cell-free plasma was then prepared and fractionated by sequential ultracentrifugation steps into VLDL (density < 1.006 gm/ml), LDL (density = 1.006 to 1.063 gm/ml), HDL (density = 1.063 to 1.21 gm/ml), and apoproteins (density > 1.21 gm/ml; plasma proteins remaining after sequential removal of lipoproteins). Endotoxin distributions are expressed as mean ± SD. 100% = total cpm LPS in cell-free plasma.

of ^{125}I-labeled LPS alone or of LPS in the presence of Hb or HSA. The majority of counts per minute were associated with the cell-free plasma in each group (Table I). Counts per minute associated with the cellular compartment, which was very small at the initial 1-minute time period, decreased even further during the 30-minute period after ^{125}I-labeled LPS injection ($p = 0.0001$). The distribution of ^{125}I-labeled LPS also was determined in whole blood, obtained before and after infusions of proteins, and then incubated with LPS in vitro. The distributions in vitro were similar to the results of the initial in vivo sample obtained at 1 minute (Table I).

Distribution of ^{125}I-labeled LPS in the cellular compartment. The distribution of ^{125}I-labeled LPS within the cellular compartment demonstrated that the majority of counts per minute localized to blood cells were associated with the platelets and erythrocytes at 1 minute after LPS injection (Table II). After 30 minutes, the percent counts per minute associated with platelets decreased ($p = 0.01$), whereas the percent counts per minute associated with erythrocytes increased ($p = 0.007$). However, there were no differences between the three experimental groups. The distribution of ^{125}I-labeled LPS was also determined in whole blood obtained before and after infusions of proteins and then incubated with LPS in vitro. The cellular distributions similarly demonstrated that the majority of LPS was associated with platelets and erythrocytes (Table II).

Distribution of ^{125}I-labeled LPS among the components of cell-free plasma. Cell-free plasma was fractionated by sequential ultracentrifugation steps into VLDL, LDL, HDL, and apoproteins. The distribution among these components, after intravenous administration of ^{125}I-labeled LPS, is shown in Table III. Fifty percent of LPS associated with cell-free plasma was observed in apoproteins, followed by LPS in the HDL fraction, in each group. There was a significant increase in the proportion of ^{125}I-labeled LPS associated with the

% PMNs			% RBCs		
Control	HSA	Hb	Control	HSA	Hb
2.0 ± 1.3	1.6 ± 1.7	0.7 ± 0.5	49.1 ± 4.0	68.5 ± 15.0	54.0 ± 19.8
1.6 ± 1.0	0.8 ± 0.7	0.8 ± 0.5	68.2 ± 1.1	76.6 ± 18.4	84.0 ± 7.9
2.9 ± 2.6	1.1 ± 2.3	3.5 ± 3.9	69.9 ± 17.8	78.5 ± 20.6	86.9 ± 7.5
4.6 ± 1.2	0.2 ± 0.4	2.2 ± 0.9	66.3 ± 19.4	87.8 ± 17.6	79.1 ± 6.1
4.6 ± 4.5	3.3 ± 2.2	3.1 ± 2.5	61.8 ± 29.1	81.8 ± 21.7	73.0 ± 6.6
5.3 ± 6.7	7.6 ± 10.6	2.8 ± 1.2	66.3 ± 15.1	47.9 ± 28.4	53.4 ± 13.5
ND	3.3 ± 2.2	1.9 ± 1.8	ND	40.8 ± 2.9	42.9 ± 9.4

Endotoxin distribution (% total cpm in plasma)					
HDL			Apoproteins		
Control	HSA	Hb	Control	HSA	Hb
36.6 ± 2.3	35.2 ± 0.6	36.6 ± 2.3	50.6 ± 0.8	53.9 ± 1.2	51.0 ± 2.3
34.9 ± 3.1	33.9 ± 2.5	35.1 ± 2.8	51.4 ± 1.4	54.3 ± 3.2	51.4 ± 3.5
37.2 ± 4.2	36.8 ± 1.2	37.7 ± 4.3	49.1 ± 2.3	50.7 ± 1.1	49.1 ± 2.9
40.2 ± 1.7	38.1 ± 3.2	39.2 ± 2.3	46.1 ± 1.3	50.9 ± 3.7	48.0 ± 2.1
40.4 ± 3.5	39.6 ± 5.4	40.8 ± 3.2	45.6 ± 0.8	49.1 ± 6.2	45.7 ± 1.8
43.5 ± 1.3	45.5 ± 1.7	42.6 ± 0.9	43.0 ± 1.6	43.2 ± 2.1	45.0 ± 1.0
ND	42.7 ± 2.5	41.0 ± 2.2	ND	46.4 ± 2.9	46.7 ± 3.9

HDL fraction ($p = 0.0006$) and a significant decrease in the apoprotein fraction ($p = 0.002$) at 30 minutes after the administration of ^{125}I-labeled LPS. The percent distribution of ^{125}I-labeled LPS in the LDL fraction also increased significantly ($p = 0.02$). However, there were no significant differences between the three experimental groups. The distribution of ^{125}I-labeled LPS in plasma after the in vitro incubation of LPS with rabbit whole blood obtained before and after protein infusion is also shown in Table III. These values were similar to the in vivo distributions obtained 30 minutes after injection of LPS.

Distribution of ^{125}I-labeled LPS in tissues. The total accumulation of LPS in 6 organs (liver, kidney, lung, spleen, adrenal, and heart) was 10.1 ± 2.9 μg in the control group, 10.6 ± 2.5 μg in the HSA group, and 12.3 ± 1.2 μg in the Hb group, respectively (not significantly different). Collectively, these values were approximately 34% ± 7% of the injected dose of LPS. Distributions of tissue-bound LPS are shown in Fig. 4, *A*. The liver contained 73.8% ± 4.5% of the radioactivity in these 6 organs. In the Hb group, spleen-bound LPS was significantly less than in the HSA group ($p = 0.05$). The concentrations of ^{125}I-l beled LPS in these organs (cpm/gm tissue) are show in Fig. 4, *B*. Liver, kidney, lung, and spleen co tained high concentrations of LPS in all three group However, in the Hb group, the concentration of ^{12} labeled LPS in the spleen was significantly lower th that in the HSA group ($p = 0.05$).

DISCUSSION

This investigation was designed to elucidate pc sible mechanisms for the previously reported synerg tic toxicity between endotoxin and Hb.[6,7,10] We co sidered that the physiologic response to LPS could altered in the presence of Hb and specifically exar ined for (1) modification of intravascular clearance endotoxin, (2) alteration of distribution of endoto among the blood cellular and plasma components, a (3) changes in the distribution of endotoxin among t major organs when LPS was administered in the pr ence of circulating Hb. The radiolabeling of LPS w ^{125}I was selected because of the reported stability LPS biophysical and biologic properties in vitro and vivo.[27,35] To provide additional evidence that the ^{12}

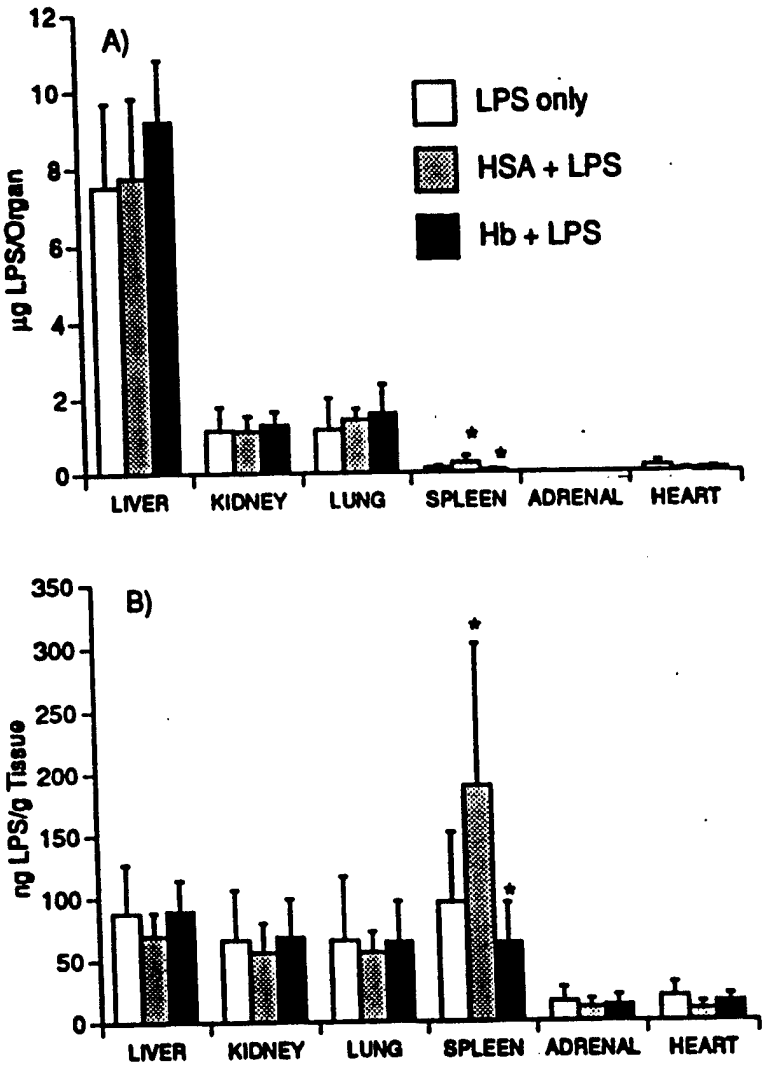

Fig. 4. The distribution of ^{125}I-labeled LPS in organs (A) and the concentrations of LPS per gram of tissue (B) after intravenous injection of 13 μg/kg of LPS into rabbits. □, LPS only; ▨, HSA plus LPS; ■, Hb plus LPS. The numbers of animals in each group were 6, 6, and 5, respectively. Values represent the mean ± SD. ★, $p < 0.05$.

labeled LPS was stable in vivo, we examined the LPS by gel permeation chromatography before and after injection. Chromatographic analysis demonstrated that there was no free ^{125}I and no evidence for LPS degradation. To confirm that the measurement of isotopic levels accurately represented the clearance of biologically active LPS, we compared the decrease in LPS concentration in blood determined by both isotopic measurements and *Limulus* test assays for biologic activity. We found good agreement between these two methods, indicating that the radiolabeled product was representative of the biologically active LPS.

The presence of Hb in the circulation significantly delayed the clearance of LPS. Our observations thus provide a possible explanation for the synergistic toxicity of LPS and Hb, based on the persistence of LPS in the circulation and the resultant additional time provided for the interaction of LPS with blood cells and endothelial cells to generate secondary messengers of endotoxemia. In contrast to our findings, Crowley et al.[36] reported that administration of Hb did not alter either clearance of injected live bacteria or levels of plasma endotoxin during bacteremia in a canine model. In their study, intravenous administration of *Escherichia coli* resulted in plasma endotoxin levels 5 to 20 times greater than in our protocol, and the elevated levels of endotoxin persisted during the 4-hour period of observation. Therefore, LPS clearance mechanisms may have differed in the two experimental models. It is possible that additional insights into

potential Hb toxicity would result from a detailed investigation of the effect of Hb on LPS clearance as a function of LPS concentration.

LPS injection caused a rapid decrease of both WBC and platelet levels, as reported previously.[20-22] However, there were no statistical differences between the experimental groups. The distribution of LPS, immediately after injection, between cellular and plasma components and among the constituents of each compartment, was similar to the results obtained after in vitro incubation of LPS with whole blood, as reported previously[19] and also shown in this study. However, in vivo, LPS associated with cells decreased significantly during the 30-minute period after injection, in part due to the occurrence of thrombocytopenia and leukopenia, although this distribution pattern was not altered by the administration of Hb.

The analysis of LPS distribution in cell-free plasma was pertinent, especially for HDL, because endotoxin bound to HDL is known to have decreased bioactivity, mainly because of a reduction of generation of important cytokines such as TNF or IL-1.[37,38] Serial analysis in our study demonstrated that the percent distribution of LPS in the HDL fraction at 30 minutes after injection was significantly higher than the value at 1 minute, and the fraction in apoproteins was significantly lower. This may be due to the redistribution of LPS over time or to the different clearance rates of LPS bound to HDL as compared with that bound to the other plasma components. This temporal in vivo alteration in distribution of LPS in cell-free plasma may reflect one of the mechanisms for detoxification of LPS in the host. Although Hb had no effects on these alterations, it is possible that the physiologic consequences of endotoxemia could be different in the presence or absence of Hb. Both Hb and HSA bind LPS, but LPS-Hb complexes are known to be more biologically active than LPS-HSA complexes[12,39]; for example, LPS-Hb complexes cause enhanced activation of *Limulus* amebocyte lysate as compared with LPS alone or LPS-HSA complexes.

The distribution and concentration of tissue-bound LPS were similar to the results reported by others.[35] An interesting finding was that rabbits that had previously received Hb had a lower accumulation of LPS in the spleen than those that had received HSA. This may be due to the blocking effect of Hb in the splenic reticuloendothelial cells, because the spleen is one of the main clearance sites for hemoglobin.[40] Because the total LPS in the spleen of rabbits was minimal as compared with that in the liver, it is unlikely that decreased splenic function accounts for the reduced clearance of LPS in the Hb group. However, this blocking effect of Hb on splenic function may cause enhanced pathophysiologic effects in human patients when endotoxemia occurs during conditions associated with reduced hepatic reticuloendothelial function, such as acute hepatitis, cirrhosis, and liver failure.[41] The reduced clearance of LPS in the presence of Hb may explain, in part, the phenomenon of high morbidity and mortality from bacterial infections in patients with sickle cell disease who have severe chronic hemolysis and absence of splenic function.[42]

In conclusion, reduced LPS clearance in the presence of Hb may contribute, in part, to the synergistic toxicity that has been reported to occur after the coadministration of LPS and Hb. Because higher doses of Hb than utilized in the current investigation will be required for resuscitation purposes, the potential for enhancement of the pathophysiologic alterations produced by concomitant endotoxemia would probably be increased. Future human trials of Hb-based blood substitutes should be designed to carefully detect LPS-related adverse reactions and should be limited, at least initially, to selected patients.[43-45]

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Effects of Bacterial Endotoxin on Human Cross-Linked and Native Hemoglobins†

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ABSTRACT: Previous investigations have demonstrated that hemoglobin (Hb) is a binding protein for bacterial endotoxin (lipopolysaccharide, LPS) and that the structure and biological activity of LPS are altered in the presence of Hb. In the present study, the influence of LPS on the structure of native human HbA_0 and covalently cross-linked Hb (αα-Hb) was studied by analyzing the absorption and circular dichroic spectra of Hb in the wavelength region of 200−650 nm. Incubation of oxyHb with each of several LPSs resulted in a decrease in the intensity of the major Soret band at 414 nm with a shift in the maximum peak to 410 nm, decreases in the intensities of the major visible region peaks at 541 and 577 nm, and the appearance of increased absorbance in the visible region in the range of 630 nm. The resultant spectra are characteristic of methemoglobin formation. These spectral changes were time-dependent and LPS-concentration-dependent. Production of methemoglobin was prominent with chemically modified, partially deacetylated rough LPS, and was observed to a lesser extent both with native, complete rough and with native smooth LPSs. The influence of LPS on the absorption spectrum of methemoglobin also was directly tested. The conversion of methemoglobin to hemichrome in the presence of LPS was demonstrated and was shown to be reversible. Analysis of circular dichroic spectra of Hb demonstrated LPS-induced spectral changes in the visible and Soret regions consistent with the production of a substantial quantity of metHb, but did not demonstrate any alteration in the far-UV region (210−240 nm). Moreover, Hb oxygen affinity was only slightly altered after incubation with any of several LPSs. In conclusion, analyses of absorption and circular dichroic spectra reveal the potential of LPS to produce a facilitated oxidation of both αα-cross-linked human Hb and native human HbA_0, without substantial changes in the secondary structure of the globin.

Purified cell-free human hemoglobin (Hb)[1] is being evaluated as a substitute for the transfusion of red cells. However, in vivo toxicities remain a major limitation for the clinical use of Hb, and a role for bacterial endotoxin (lipopolysaccharide, LPS) in the observed toxicity has been proposed (Roth & Kaca, 1994). We recently have shown that purified, native human hemoglobin (HbA_0) or a chemically cross-linked human hemoglobin (αα-Hb) forms complexes with bacterial endotoxin (LPS) (Kaca et al., 1994a), and that complex formation significantly enhances deleterious biological activities of LPS in vitro, including activation of coagulation (Kaca et al., 1994b), stimulation of mononuclear cell tissue factor production (Roth et al., 1993), and transformation of endothelial cells into a procoagulant surface (Roth, 1994). In vivo, Hb and LPS have been demonstrated by others to synergistically stimulate coagulation, complement proteolytic cascades, and increase lethality (White et al., 1986; Feola et al., 1988a,b). Comparisons of several chemically distinct LPSs, including chemically modified species that allow detailed investigation of LPS structure/function relationships, have identified LPS chemical moieties that are critical for this interaction (Kaca et al., 1994b). Hb/LPS complex formation also was associated with a decrease in the aggregation state of LPS, i.e., a decrease in size and density of the LPS macromolecular structure (Kaca et al., 1994a), and we proposed that this alteration in LPS structure may constitute a physiologically significant process when Hb and LPS coexist intravascularly. The affinity of Hb for LPS has been shown to be high (Kaca et al., 1994a), and unexpectedly high levels of LPS contamination of Hb solutions have been demonstrated (Roth et al., 1993), thus making the in vivo interaction of Hb and LPS of considerable concern for the development of this potential red cell substitute.

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[1] Abbreviations: hemoglobin, Hb; bis(3,5-dibromosalicyl) fumarate αα-cross-linked hemoglobin, αα-Hb; αα-cross-linked methemoglobin, metαα-Hb; bacterial lipopolysaccharide, LPS.

Cell-free Hb is particularly susceptible to oxidation and denaturation, and Hb or its degradation products, heme or iron, can act as Fenton reagents to promote hydroxyl radical formation (Sadrzadeh et al., 1984). In animals, Hb infusion has been shown to result in the generation of reactive oxygen compounds (Feola et al., 1988b) and has been associated with lipid peroxidation and with organ failure (Paller, 1988). These deleterious consequences of Hb infusion can be reduced by iron chelation, suggesting that the instability of cell-free Hb, the release of iron, and the resultant production of toxic oxygen- and lipid-derived free radicals may be responsible for some of the observed in vivo deleterious consequences of Hb infusion (Feola et al., 1988a; Paller, 1988; Faasen et al., 1988; Simoni et al., 1990). In addition, the presence of Hb, or free iron which is released from Hb during the process of Hb oxidation and denaturation, enhances mortality associated with Gram-negative infection (Eaton et al., 1982), and both free iron and free heme potentiate oxidant-mediated cell damage (Faasen et al., 1988; Balla et al., 1991). Therefore, an important aspect of the development of cell-free Hb solutions has been the evaluation of various derivatized Hb preparations for enhanced stability.

Although our previous studies have demonstrated that Hb binding has a major influence on LPS structure and function, it was not known whether this interaction reciprocally changes the conformation or stability of Hb. However, it has been reported that methemoglobin and oxyhemoglobin are converted to hemichrome in the presence of fatty acids (Akherm et al., 1989), and since fatty acids are one of the main components of LPS (Rietschel et al., 1991), we thought it possible that LPS could similarly alter Hb. In order to assess whether LPS can induce protein structural changes in Hb leading to its oxidation, we studied the abilities of several purified and chemically characterized LPSs to alter the absorption and circular dichroic spectra of both native and chemically modified human Hb.

MATERIALS AND METHODS

Reagents. Human serum albumin (HSA) (25%, for injection) was purchased from Nybcen (New York, NY). Sterile, endotoxin-free 0.9% NaCl was purchased from Travenol Laboratories (Deerfield, IL). Xylenol orange (*o*-cresolsulfonephthalein-3′,3″-bis(methyliminodiacetic acid), sodium salt) was obtained from the Aldrich Chemical Co. (Milwaukee, WI); butylated hydroxytoluene and ammonium ferrous sulfate were obtained from Sigma (St. Louis, MO).

Hemoglobin. Human hemoglobin was prepared and purified, as described previously (Winslow et al., 1995), by the Blood Research Division at the Letterman Army Institute of Research, San Francisco, CA. Human hemoglobin was covalently cross-linked between Lys 99 residues of the α subunits (ααHb) with bis(3,5-dibromosalicyl) fumarate (DBBF). The ααHb stock solution was 9.6 g/dL, pH 7.4, in Ringers acetate and contained less than 0.4 EU/mL endotoxin (referenced to *Escherichia coli* lipopolysaccharide B, O55:B5, Difco Laboratories, Detroit, MI), as determined by the Limulus amebocyte lysate (LAL) test (Levin & Bang, 1968). The ααHb stock solution was stored at −70 °C and then diluted with sterile, pyrogen-free 0.9% NaCl prior to use. Purified non-cross-linked human hemoglobin A_0 (HbA_0), 8.4 g/dL, was prepared from Hb by ion-exchange HPLC (Christensen et al., 1988). All hemoglobin concentrations are given on a per heme basis.

Methemoglobin (metααHb) was prepared by oxidation of ααHb with potassium ferricyanide (Di Iorio, 1981). The reaction was carried out at 4 °C in the dark for 30 min with occasional mixing. The molar ratio of $K_3Fe(CN)_3$ to ααHb was 1.2:1. MetααHb was separated from ferrocyanide and residual ferricyanide by Sephadex G-25 M (PD-10, Pharmacia, Piscataway, NJ) column chromatography. The methemoglobin stock solution (3 mg/ml) was stored at 4 °C in 0.05 M potassium phosphate (pH 7.4) and was used within 2 weeks.

Bacterial Endotoxin (Lipopolysaccharide, LPS). Smooth LPSs were extracted with phenol−water according to the Westphal method (Westphal & Jann, 1965), whereas rough LPSs were extracted by the phenol−chloroform−petroleum ether (PCP) method of Galanos (Galanos et al., 1969). Crude LPSs were further purified by sequential treatment with RNAse and DNAse, followed by ultracentrifugation at 100000*g* for 3 h, as described previously (Kaca et al., 1987). All LPSs were essentially free of nucleic acids (indicated by a lack of absorbance at 260 nm), and contamination by proteins was less than 2% on the basis of BCA protein assays.

Crude *E. coli* 026 (smooth) LPS was purchased from Difco and then further purified as described above. *Proteus mirabilis* O3 smooth strain LPS was provided by collaborators at the Institute of Microbiology and Immunology, University of Lodz, Poland, and was purified as described above. The deep rough *Salmonella minnesota* 595 LPS, Re type, was extracted by the PCP method (Galanos et al., 1969) and then further purified. *S. minnesota* 595 lipid A was prepared from 134 mg of Re 595 LPS by hydrolysis of the ester-bound ketodeoxyoctulosonic (KDO) residue with sodium acetate buffer (pH 4.4) for 1 h at 100 °C (Brade et al., 1983). The hydrolysate was dialyzed to obtain purified lipid A (67% recovery from the Re 595 starting material) and then lyophilized and stored at 4 °C. KDO (83.5%) from LPS 595 was released by this procedure, as determined colorimetrically using thiobarbituric acid. Singly deacetylated *S. minnesota* 595 LPS (OH37 LPS) was produced from 380 mg of Re 595 LPS by hydrolysis of a single ester-bound 3-hydroxytetradecanoyl fatty acid from the reducing glucosamine of LPS with 0.2 N NaOH for 30 min at 37 °C (Myers et al., 1990). The hydrolysate then was cooled to 4 °C and neutralized to pH 6.5 with 0.1 N HCl. Released fatty acids were extracted by $CHCl_3$/MeOH (2:1) followed by precipitation of OH37 LPS by EtOH/acetone (2:1) at 4 °C. The OH37 LPS sediment was centrifuged at 10000*g* for 30 min, washed twice with cold EtOH, resuspended in water, and lyophilized (61% recovery of the starting material was obtained). One hundred percent removal of 3-hydroxytetradecanoyl fatty acid was demonstrated by gas−liquid chromatography (GLC) analysis of methyl esters of fatty acids isolated from OH37 LPS.

Absorption Spectra of Hemoglobin in the Presence of Endotoxin. Solutions of LPS and Hb were prepared in 0.05 M Tris buffer, pH 7.4, in phosphate-buffered saline (PBS), pH 7.4, or in endotoxin-free 0.9% NaCl. The Hb/LPS mixtures were incubated at 37 °C for various periods of time, and Hb absorption spectra (between 350 and 730 nm) were then recorded using either a Lambda 3B (Perkin-Elmer, Norwalk, CT) or an upgraded Cary-14 (On-Line Instrument Systems, Bogart, GA) UV/vis spectrophotometer. The percentages of oxyHb, metHb, and hemichromes were

determined by the method described by Winterbourn (Winterbourn, 1990). Each experiment was performed at least three times, and representative data are presented.

Circular Dichroism. Circular dichroic (CD) spectra of Hb and Hb/LPS complexes were measured with a Jasco J-500A spectropolarimeter (Japan Spectroscopic Company, Tokyo, Japan). Samples were measured in 50 mM potassium phosphate, pH 7.4. The data are expressed as molar ellipticity on the basis of heme concentration. α-Helical content was estimated from the mean residue ellipticity at 222 nm on the basis of amino acid residues according to the reference value of $[\theta]_{222} = -30\,300f_H - 2340$ (Chen et al., 1972), where f_H represents the fraction of α-helix.

Oxygen Affinity Measurements. P_{50} values were determined for Hb and Hb/LPS complexes from O_2 equilibrium curves obtained with a Hemox-Analyzer (TCS Medical Products, Philadelphia, PA). Samples were diluted in Hemox buffer (pH 7.4) provided by the manufacturer.

Measurement of Peroxides and Oxidants. Peroxides or other oxidizing agents were determined with the ferrous oxidation/xylenol orange (FOX) reagent, prepared as described previously (Jiang et al., 1992). Briefly, ammonium iron (Fe^{2+}) sulfate, xylenol orange, and butylated hydroxytoluene were dissolved in 90% methanol/10% sulfuric acid to produce the FOX reagent. Various concentrations of ααHb alone or ααHb and OH37 LPS in endotoxin-free 0.9% NaCl were incubated at 37 °C for 60 min, and then 2 mL of FOX reagent was added to 0.2 mL of each sample. Following an additional 30-min incubation at room temperature, absorbances of Fe^{3+}−xylenol orange complexes were measured at 570 nm with a Lambda 3B UV/vis spectrophotometer (Perkin-Elmer, Norwalk, CT). Samples were assayed in duplicate or triplicate, and mean values are presented.

RESULTS

Absorption Spectroscopy. Changes in the absorption spectra of ααHb and HbA_0 resulting from their interaction with LPS were examined with three chemically distinct enterobacterial LPSs (Figure 1). In the presence of LPSs, HbA_0 (Figure 1A) and ααHb (Figure 1B) each underwent substantial spectral alterations, characterized by decreases in the major visible region peaks at 541 and 577 nm and the appearance of an increased absorbance in the range of 630 nm. Also noted with each LPS was a decrease in the intensity of the major Soret peak of oxyHb at 414 nm (data not shown in Figure 1; see Figure 2). These spectral changes are characteristic of ferric Hb species (Winterbourn, 1990). The spectral changes were most prominent with deacetylated OH37 LPS (curve 4), with lesser changes induced by 595 LPS (curve 3) and 03 LPS (curve 2). The spectral changes in HbA_0 induced by OH37 LPS were slower in Tris buffer, pH 7.4 (Figure 1C, curve 4) than in 0.9% NaCl at pH 6.5 (Figure 1A), although similar spectral changes were eventually observed (after 18 h) at pH 7.4 (Figure 1D, curve 4). The pH of buffered incubations (pH 7.4) remained constant during the study; incubations performed in 0.9% NaCl showed slight pH differences between Hb alone (pH 6.5) and Hb/LPS mixtures (pH 6.5−6.8). The reduction of absorbance in the range of 630 nm to less than control (Hb alone, line 1), observed with 595 LPS/HbA_0 or 595 LPS/ ααHb (Figure 1A−D, line 3), suggests that both Hbs increased the solubility of 595 LPS and therefore decreased its light scattering, in comparison to the light scattering by the less soluble 595 LPS sample in the reference cuvette.

Because the most prominent Hb spectral alterations were observed after incubation with OH37 LPS, we further characterized this interaction by investigating the time course (Figure 2 and 3) and LPS concentration dependence (Figure 4) of these spectral changes. These experiments were done in PBS (pH 7.4) to maintain constant pH and maximize Hb stability during the course of the reaction. In the Soret region, a time-dependent progressive loss of intensity of the major Soret peak at 414 nm was associated with a shift in the peak maximum to 410 nm (Figure 2A). Progressive decreases in the major visible region peaks at 541 and 577 nm (Figure 2B) also were observed over a 120-min time period and were accompanied by the appearance of a gradual increase in absorbance in the range of 630 nm.

From mathematical evaluation of these changing spectra (as described in Materials and Methods), the percentage of oxyααHb was shown to be substantially decreased (from 90% initially to 30%) after incubation with OH37 LPS for 120 min, and the calculated percentages of metααHb and hemichromes each were increased from 4% initially to 37% and 30%, respectively, after incubation for 120 min with OH37 LPS (Figure 3A, closed symbols). Time-dependent changes of the spectrum of native HbA_0 in the presence of OH37 LPS also were recorded, demonstrating alterations similar to those observed with ααHb. Increased proportions of $MetHbA_0$ and hemichromes to 35% and 39%, respectively, after a 60-min incubation at 37 °C accompanied a decrease in $oxyHbA_0$ to 26% (Figure 3B, closed symbols). Production of metHb preceded the production of hemichromes. In contrast to the prominent Hb oxidation forms induced by OH37 LPS, incubation of ααHb or HbA_0 in the absence of LPS resulted in minimal Hb oxidation (Figure 3A,B, open symbols).

The rate of conversion of oxyHb to metHb and hemichromes was shown to be dependent on LPS concentration (Figure 4). After incubation of ααHb with OH37 LPS for 1 h, spectral changes in the Soret and visible regions similar to those shown in Figure 2 were observed to be greatest at 1 mg/mL LPS, but were also produced by 0.5 mg/mL and 0.05 mg/mL LPS (spectra not shown for Figure 4). Apparent isosbestic points were present at 381 and 437 nm in the Soret region, and at 523 and 589 nm in the visible portion of the spectrum (spectra not shown). On the basis of mathematical evaluation of these spectra, LPS concentration-dependent conversion of ααHb to metααHb and hemichromes was demonstrated (Figure 4A). At the highest concentration of OH37 LPS studied (1 mg/ml), nearly all of the starting oxyααHb (89%) was converted to metααHb and hemichromes (46% and 41%, respectively) after 1 h. Similar to ααHb, LPS dose-dependent spectral changes were recorded for HbA_0 (data not shown), and nearly all of the $oxyHbA_0$ was converted to $metHbA_0$ (41%) and hemichromes (32%) after 1 h (Figure 4B). The spectral changes of ααHb and HbA_0 in the presence of various concentrations of OH37 LPS were also tested in unbuffered 0.9% NaCl, and similar extents of denaturation were observed (data not shown).

Since the time-dependent production of metHb from oxyHb in the presence of LPS preceded the production of hemichromes (Figure 3), we directly examined the ability of metHb to form hemichromes. MetααHb, produced as

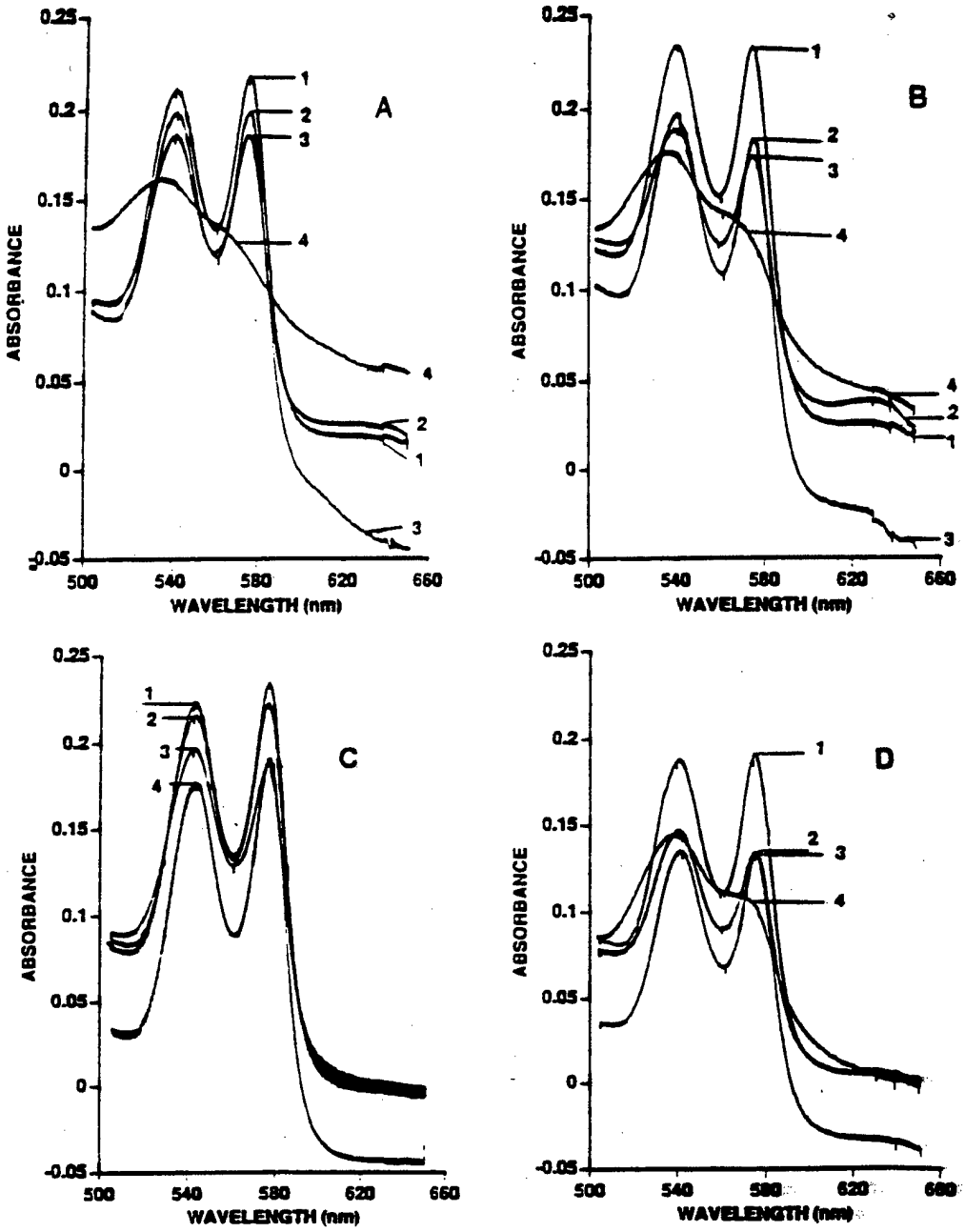

FIGURE 1: Hemoglobin absorption spectra in the absence and presence of LPS. Native oxyhemoglobin (HbA_0, 15 μM) and cross-linked oxyhemoglobin ($\alpha\alpha$Hb, 16 μM) preparations were incubated with each of several LPSs (each 1 mg/mL) at 37 °C, and Hb absorption spectra in the visible region were recorded. Hb in the absence of LPS is shown as line 1 in each graph. LPSs examined included *P. mirabilis* O3 LPS (line 2), *S. minnesota* 595 LPS (line 3), and *S. minnesota* 595 OH37 LPS (line 4). (A) Incubations with HbA_0 for 1 h in 0.9% NaCl. (B) Incubations with $\alpha\alpha$Hb for 1 h in 0.9% NaCl. (C) Incubations with HbA_0 for 1 h in 0.05 mM Tris buffer, pH 7.4. (D) Incubations with HbA_0 for 18 h in 0.05 mM Tris buffer, pH 7.4. In each experiment, the sample cuvette contained Hb with or without LPS, and the reference cuvette contained 0.9% NaCl (for Hb spectra alone) or LPS alone (1 mg/mL in 0.9% NaCl) (for Hb/LPS mixture spectra).

described in Materials and Methods, was incubated with OH37 LPS, and spectra were obtained (Figure 5). After the reaction was completed, the mixture of met$\alpha\alpha$Hb plus OH37 LPS (1 mg/mL) had a pH of 6.6; the pH of met$\alpha\alpha$Hb alone was 6.3. A time-dependent (Figure 5B) and LPS concentration-dependent (Figure 5C) increase in absorbance at 537 nm and decrease in absorbance of the prominent met$\alpha\alpha$Hb peak at 630 nm were observed, indicating transformation from high-spin met$\alpha\alpha$Hb to low-spin hemichromes. After a 10-min incubation with OH37 LPS (which generated curve 3 in Figure 5B), the Soret peak was not appreciably altered (Figure 5A). However, more prolonged incubation demonstrated a decrease in the intensity of the major Soret peak and a shift of the peak maximum from 405 to 411 nm (Figure 6, described below).

Since production of hemichromes from oxyHb can be either reversible or irreversible, we examined whether the addition of albumin, which forms complexes with LPS, allowed re-formation of met$\alpha\alpha$Hb from the hemichrome/LPS mixture (Figure 6). Human albumin was used because albumin is a well-characterized LPS binding protein (Galanos et al., 1972) and therefore had the potential ability to bind LPS already bound to hemichromes. Met$\alpha\alpha$Hb (Soret peak absorbance at 405 nm, Figure 6, spectrum 1) was first

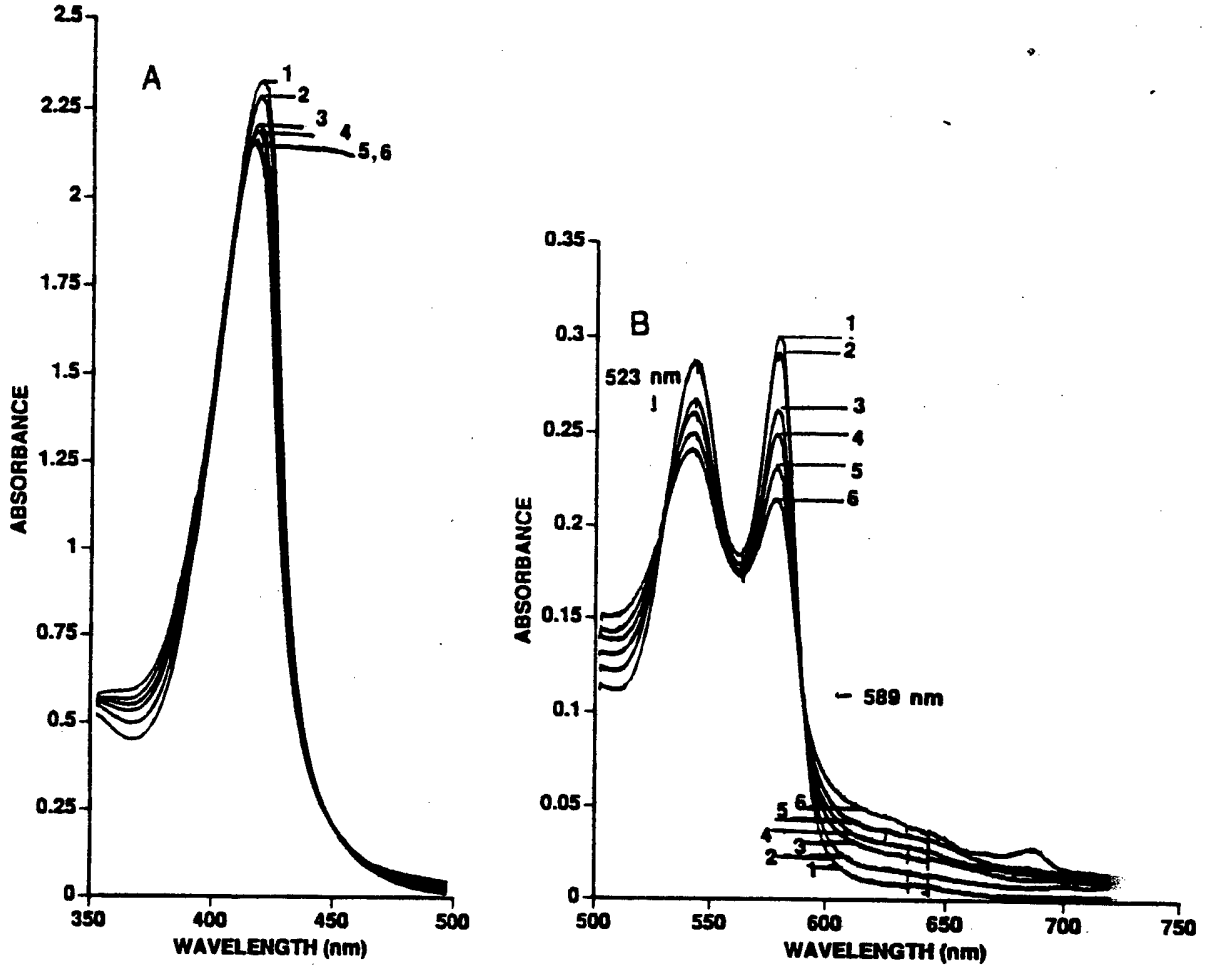

FIGURE 2: Time course of changes in the hemoglobin absorption spectrum in the presence of LPS. ααHb (21 μM in PBS, pH 7.4) was incubated at 37 °C in the presence of 0.3 mg/mL *S. minnesota* 595 OH37 LPS, and absorbance spectra in the Soret (A) and visible (B) regions of the Hb spectrum were obtained at various times of incubation. Line 1, initial spectrum of ααHb alone; line 2, 10 min; line 3, 20 min; line 4, 40 min; line 5, 90 min; line 6, 120 min. The sample cuvette contained Hb in PBS with or without LPS, and the reference cuvette contained PBS (for ααHb spectra alone) or LPS alone (0.3 mg/mL in PBS) (for ααHb/LPS mixture spectra). The arrows indicate the apparent isosbestic points.

incubated with OH37 LPS to produce hemichromes (Soret peak absorbance at 411 nm, Figure 6, spectrum 2). Human albumin then was added to the hemichrome/LPS mixture, and a shift of the Soret peak maximum from 411 nm back to 405 nm was subsequently observed (Figure 6, spectrum 3). These results are consistent with transformation of low-spin hemichromes back to high-spin metααHb, suggesting that the process of hemichrome formation in the presence of LPS was reversible. The transformation of hemichromes to metααHb was completed within 5 min at room temperature and was associated with a decrease in the amplitude of the peak absorbance at 405 nm in the final solution, as compared to the intensity of the initial metααHb absorbance peak before hemichrome formation. The decreased intensity of the 405-nm peak in the final metααHb/LPS mixture, after addition of albumin solution, suggested either that the LPS-induced formation of hemichromes was only partially reversible when LPS was removed (although no shoulder could be detected at 411 nm in Figure 6, spectrum 3) or that the process of reversing hemichromes back to metααHb was associated with a loss of heme from hemoglobin. Heme loss may have been associated with its binding to albumin, a well-known phenomenon (Vandegriff & Le Tellier, 1994). However, in the absence of LPS, heme loss from metααHb to albumin, as indicated by a decrease in absorbance at 405 nm, was not detectable before 15 min, and there was no shift of the peak maximum absorbance from 405 to 411 nm (data not shown).

Circular Dichroic Spectroscopy. The demonstration that Hb binding of LPS resulted in heme oxidation suggested that LPS binding could initially alter globin structure and secondarily destabilize the heme pocket. To evaluate this possibility, we used circular dichroic (CD) analysis of Hb to assess the ability of LPS to affect Hb secondary structure. Initially, we studied *S. minnesota* 595 OH37 LPS because Hb oxidation by this LPS was prominent and rapid. ααHb was incubated with OH37 LPS for 2 h, after which absorbance measurements of the starting Hb and the Hb/OH37 LPS mixture indicated that oxyHb had decreased from 97% to 50% with the production of 42% metHb and 8% hemichrome. Following incubation of Hb with LPS, we observed a decrease in the intensity of the CD peak at 579 nm, a decrease in intensity and a shift to lower wavelength of the Soret peak (from 420 nm to 418 nm), and a decrease in the intensity of the near-UV peaks at 259 and 265 nm (Figure 7). These changes in the Hb CD spectrum can be accounted for by the loss of a substantial fraction of oxyHb and the concomitant production of MetHb. Compared to Hb/LPS, MetHb by itself demonstrated more prominent changes from Hb alone in this wavelength region (i.e., 250–

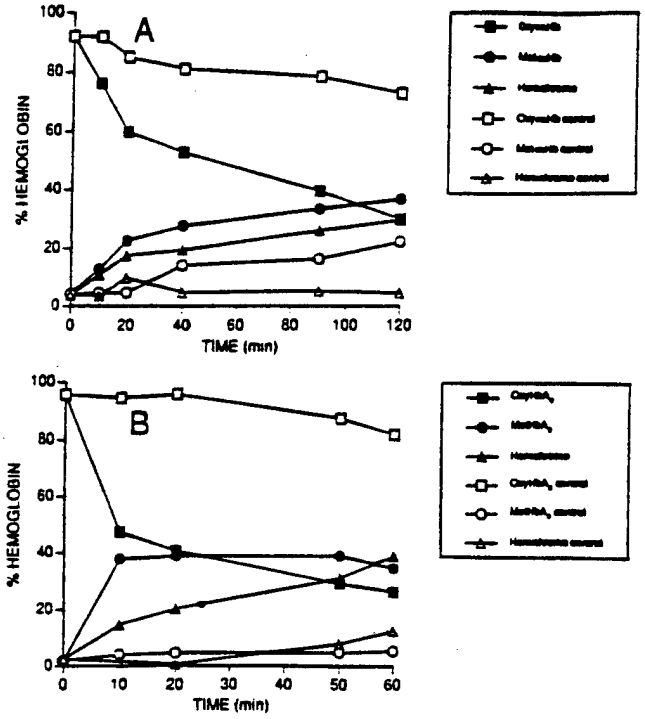

FIGURE 3: Time-dependent conversion of ααHb (A) and HbA_0 (B) to metHb and hemichromes in the presence of *S. minnesota* 595 OH37 LPS (0.3 and 0.8 mg/mL LPS incubated with ααHb and HbA_0, respectively). Percentages of oxyHb, metHb, and hemichromes were determined as described in Materials and Methods. Open symbols, Hb alone; closed symbols, Hb + LPS.

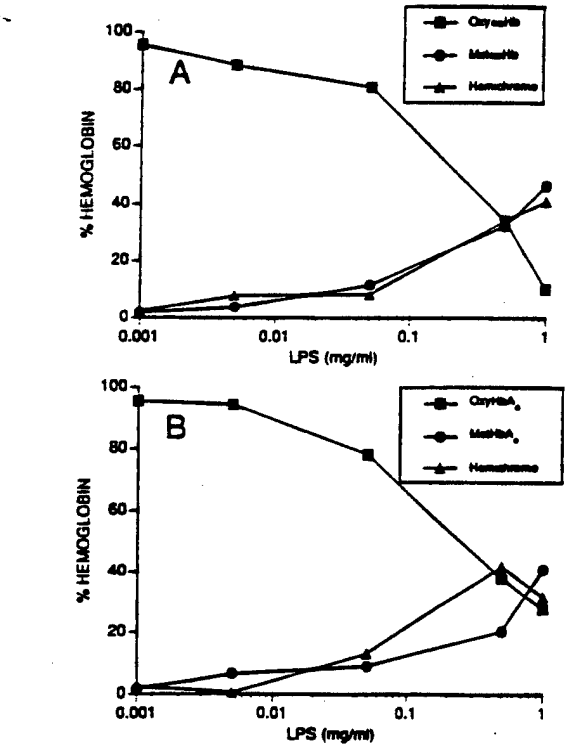

FIGURE 4: Conversion of ααHb (A) and HbA_0 (B) to metHb and hemichromes in the presence of various concentrations of *S. minnesota* 595 OH37 LPS after a 1-h incubation. Percentages of oxyHb, metHb, and hemichromes were determined as described in Materials and Methods.

600 nm), and also showed a considerable decrease in CD intensity in the far-UV region (Figure 7). In contrast, there was no important spectral change in the wavelength range of 210−240 nm after a 2-h incubation of Hb with OH37 LPS. α-Helical content was estimated to be 53% for the globin in either the absence or the presence of LPS. Therefore, although the visible and near-UV spectral data were consistent with destabilization of the heme, there was no evidence for alteration of globin structure. CD spectral changes induced by a smooth LPS (*E. coli* 026) (data not shown) were similar to those observed with *S. minnesota* 595 OH37 LPS, although an overnight incubation was required with *E. coli* 026 LPS in order to generate the altered CD spectra observed with OH37 LPS after 2 h.

P_{50} Measurements. We measured the oxygen affinity of Hb in the absence and presence of LPS in order to evaluate the possible influence of LPS binding on Hb function. These measurements were made after a 2-h incubation period, a time sufficient to result in Hb/LPS complex formation (Kaca et al., 1994a), but prior to the formation of substantial quantities of oxidized Hb species unable to bind oxygen. Hb at 1 mg/mL (16 μM) and 1 mg/mL of each LPS were utilized because the two components of Hb/LPS complexes are of approximately equal concentration by weight (Kaca et al., 1994a), and little unbound Hb is calculated to be present. P_{50} values for ααHb (26.6 mmHg) and HbA_0 (9.6 mmHg) were slightly decreased by both smooth and rough LPSs (Table 1). Non-cross-linked cell-free HbA_0, which exhibited high oxygen affinity (P_{50} = 9.6 mmHg) similar to that measured with lysed whole blood (P_{50} = 10.0 mmHg; data not shown), best demonstrated the trend toward higher oxygen affinity when in the presence of LPS (P_{50} = 7.3 mmHg in the presence of OH37 LPS).

FOX Assays for Oxidants. Because Hb oxidation is known to result in production of the free radical peroxide, and because our data indicated that LPS was capable of stimulating the production of metααHb and hemichromes, we used the FOX assay to investigate whether detectable Hb-derived oxidants were increased in the presence of LPS. The FOX assay is based on rapid peroxide-mediated oxidation of Fe^{2+} from ammonium ferrous sulfate to Fe^{3+}; the latter forms Fe^{3+}/xylenol orange complexes which are measured at 570 nm (Jiang et al., 1992). ααHb alone, at a concentration higher than 0.2 mg/mL, generated an agent that oxidized Fe^{2+} to Fe^{3+} in a concentration-dependent manner (Figure 8). The addition of 1 mg/mL OH37 LPS to Hb did not alter the concentration of detectable oxidizing products, and LPS alone did not generate oxidizing products (Figure 8, △). In confirmatory experiments, concentrations of other LPSs as great as 1 mg/mL similarly did not increase the concentration of detectable oxidizing products derived from Hb (data not shown). Addition of Hb alone or Hb plus LPS to FOX reagent, prepared without ammonium ferrous sulfate, did not produce Fe^{3+}/xylenol orange complexes; i.e., there was no detectable absorbance at 570 nm. This indicated that Fe^{3+} from Hb was not the source of the Fe^{3+} that bound to xylenol orange.

DISCUSSION

Cross-linked hemoglobin is being developed as a potential red blood cell substitute (DeVenuto & Zegna, 1982; Sehgal et al., 1984; Winslow, 1992; Vandegriff, 1993). To date, clinical use of Hb solutions has been restricted because of their toxicity, which in some instances may be related to contamination by bacterial endotoxin (Roth et al., 1993; White et al., 1986; Litwin et al., 1963). We have shown

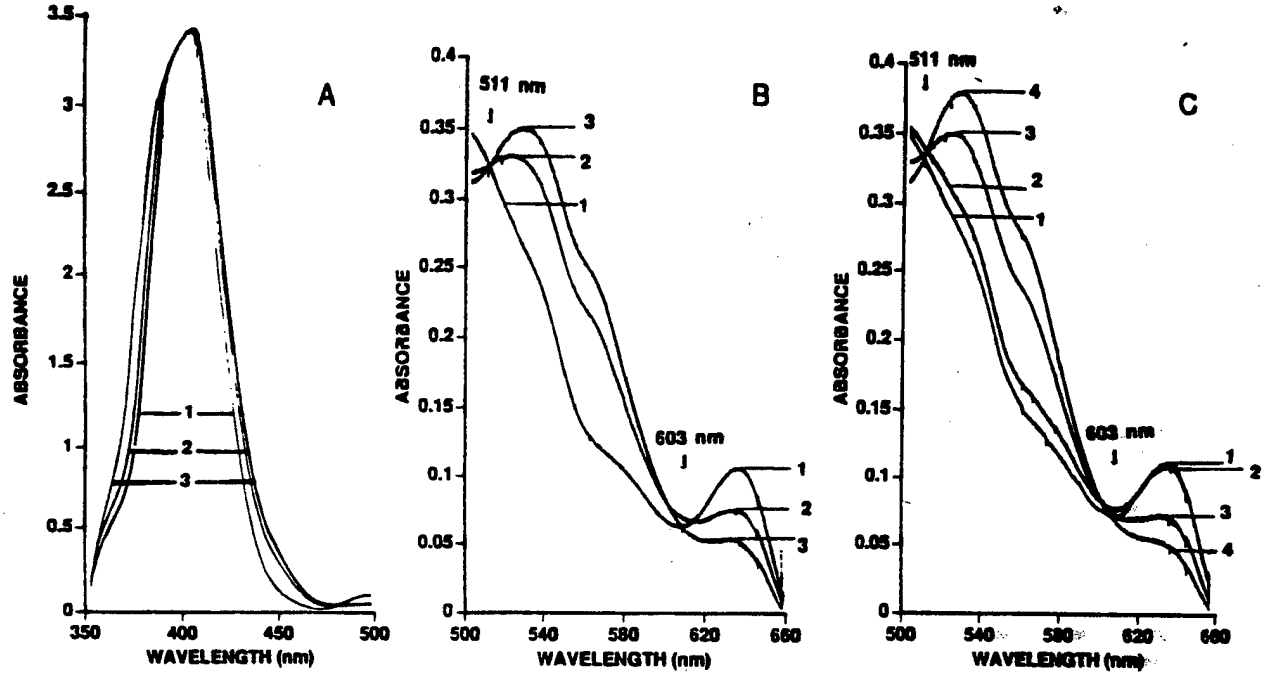

FIGURE 5: Methemoglobin absorption spectra in the absence and presence of LPS. MetααHb (24 μM), prepared from ααHb as described in Materials and Methods, was incubated at 37 °C in the absence or presence of OH37 LPS, and metααHb absorbance spectra were recorded as a function of time of incubation (A and B) or LPS concentration (C). The arrows indicate the apparent isosbestic points. Panels A (Soret) and B (visible region): line 1, initial spectra of metααHb alone; lines 2 and 3, metααHb with 1 mg/mL OH37 LPS incubated for 5 and 10 min, respectively. Panel C (visible region): line 1, metααHb alone; line 2, metααHb and 0.05 mg/mL LPS; line 3, metααHb and 0.5 mg/mL LPS; line 4, metααHb and 1 mg/mL LPS. Samples were incubated for 15 min. The sample cuvette contained metααHb with or without LPS, and the reference cuvette contained 0.9% NaCl (for metααHb spectra alone) or LPS alone at the appropriate concentration in 0.9% NaCl (for metααHb/LPS mixture spectra).

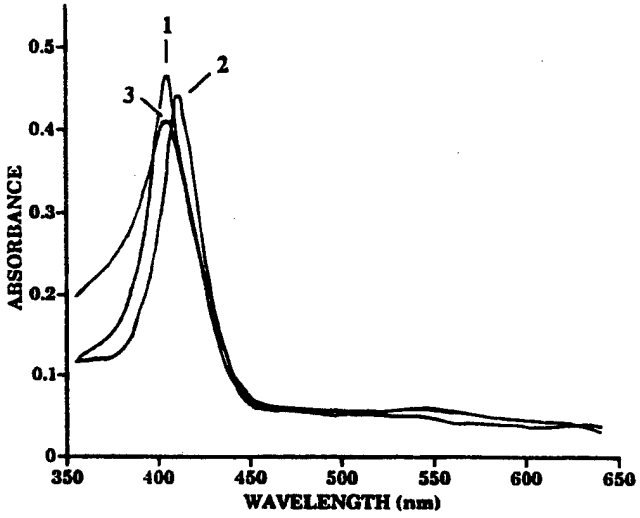

FIGURE 6: Reversibility of hemichrome formation from methemoglobin in the presence of LPS and albumin. MetααHb alone (prepared as described in Materials and Methods) (line 1) was incubated with 1 mg/mL OH37 LPS for 30 min at 37 °C (line 2). Human albumin (1 mg/mL) subsequently was added, and after 5 min at 37 °C incubation, the resulting absorbance spectrum in the Soret region was recorded (line 3). The sample cuvette contained metααHb with or without LPS or with LPS and albumin; the reference cuvette contained 0.9% NaCl (for metααHb spectra alone) or LPS alone in 0.9% NaCl (for metααHb/LPS and metααHb/LPS/albumin mixture spectra).

previously that a variety of Gram-negative bacterial endotoxins form complexes with hemoglobin (Kaca et al., 1994a,b). Furthermore, in the presence of hemoglobin, LPS is more reactive in some biological models, i.e., Limulus amebocyte activation and tissue factor production by human endothelial cells and mononuclear cells (Kaca et al., 1994a,b; Roth et al., 1993; Roth, 1994). These results indicate that hemoglobin can significantly alter the physicochemical features of LPS.

In the present study, we have shown that oxyhemoglobin may be less stable in the presence of LPS. The observed spectral changes in the Soret and visible regions of the absorption spectra of cross-linked ααHb and native HbA_0, produced by a variety of chemically different LPSs, are indicative of methemoglobin and hemichrome formation. Time- and concentration-dependent methemoglobin and hemichrome formation were most prominently demonstrated with partially deacetylated OH37 LPS. The lowest concentration of OH37 LPS demonstrating this effect was 0.05 mg/mL (18 μM on the basis of the known molecular weight of this LPS derivative). The concentration of ααHb in these experiments was 21 μM, suggesting that about 1 mol of OH37 LPS was bound per mole of Hb. The smooth *P. mirabilis* O3 and deep rough mutant *S. minnesota* 595 LPSs also were shown to oxidize hemoglobins, although less effectively than partially deacetylated LPS. The mechanism by which LPS causes Hb oxidation is not known. However, similar spectral changes in Hb have been described previously after addition of carbon-centered radicals (Minetti et al., 1993). Since fatty acids are known to be a common source of carbon-centered radicals (Akherm et al., 1989; Minetti et al., 1993; Buege & Aust, 1978), it is possible that the fatty acyl components of LPS may be a source of free radicals that in turn produce Hb oxidation.

Our data indicate that once methemoglobin is present, further incubation with LPS results in its fast conversion to hemichromes. The conversion of methemoglobin to hemichrome by LPSs was at least partially reversible with the addition of human albumin, suggesting that the binding of

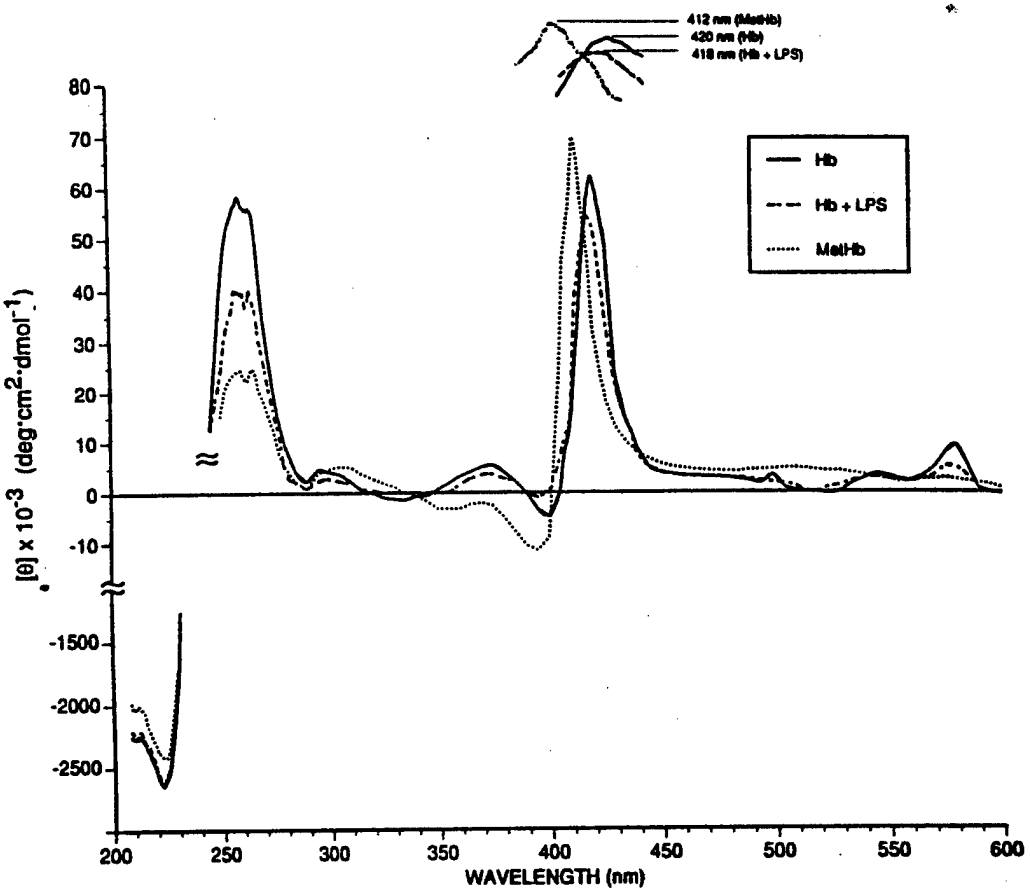

FIGURE 7: Circular dichroic (CD) spectra of Hb in the absence and presence of LPS. CD spectra were measured at room temperature between 200 and 600 nm for ααHb alone (13.8 μM heme, ambient oxygenation), ααHb/LPS (13.8 μM heme, 0.5 mg/mL *S. minnesota* 595 OH37 LPS, after a 2-h incubation at 37 °C), and metααHb (31.5 μM heme). Measurements in the far-UV region were made with samples diluted 5−10-fold. A 1-cm path length cell was utilized for measurements between 250−400 and 430−600 nm, and a 0.2-cm path length cell was utilized for measurements of the major Soret (400−440 nm) and far-UV (210−250 nm) regions. Ellipticities, [θ], are expressed on a molar heme basis. Wavelengths for the Soret peak maxima are identified on inset tracings presented with an expanded *x*-axis.

Table 1: P_{50} Values for Hb and Hb/LPS Complexes[a]

	P_{50}
ααHb alone	26.6
ααHb + LPS[b]	25.1
ααHb + LPS[c]	25.6
HbA_0 alone	9.6
HbA_0 + LPS[d]	8.7
HbA_0 + LPS[e]	7.3

[a] Oxygen affinity measurements were obtained for cross-linked (ααHb) and native (HbA_0) hemoglobins alone or in the presence of LPS after a 2-h incubation at 37 °C. Measurements were obtained prior to the production of oxidized Hb species. P_{50} was determined by utilizing both smooth and rough LPSs. Equal concentrations of Hb and LPS were utilized (each 1 mg/mL prior to dilution in Hemox buffer). [b] *P. Mirabilis* 03 (smooth) LPS. [c] *S. minnesota* Re 595 (rough) LPS. [d] *E. coli* 026 (smooth) LPS. [e] *S. minnesota* 595 OH37 (rough) LPS.

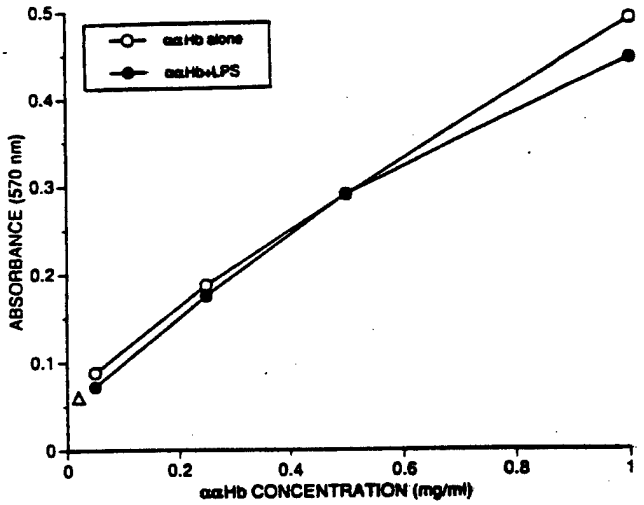

FIGURE 8: Production of free radicals in the absence and presence of LPS. OxyααHb (concentration, 0.05−1 mg/mL) was incubated at 37 °C for 60 min in the absence (○) or presence (●) of 1 mg/mL OH37 LPS. FOX reagent was then added, and oxidizing products were detected by absorbance at 570 nm. LPS alone (1 mg/mL) (△) did not produce detectable oxidants. Each point is the mean of two determinations. The data are representative of three independent experiments.

LPS to albumin is stronger than LPS binding to hemoglobin. Since hemichrome reversibility was associated with loss of magnitude of the resultant metααHb Soret peak compared to the initial spectrum, there may have been only partial reconversion of metααHb to hemichrome; alternatively, some of the heme in hemichrome may have been lost to albumin.

The superior ability of partially deacetylated OH37 LPS to facilitate methemoglobin and hemichrome formation, compared to that of the intact parent rough 595 LPS, suggests a possible biochemical mechanism for the Hb−LPS interaction. Rough LPS, suspended in aqueous solution at physiologic conditions of temperature and divalent cation concentration, is aggregated to form nonlamellar cubic structures, with its hydrophobic fatty acids facing inward and the hydrophilic carbohydrate components exposed to the external

environment (Rietschel et al., 1991). Partial deacetylation may disturb the supramolecular structure of LPS and expose the fatty acids of the lipid A component of LPS (Myers et al., 1990). One may speculate that the hydrophobic fatty acids of LPS interact with some hydrophobic domain of hemoglobin and subsequently facilitate iron oxidation and degradative processes that affect the globin protein. Detoxification of bacterial lipopolysaccharides in vivo occurs via enzymatic release of secondary acyl chains from lipid A (Munford & Hall, 1986) in a process similar to the deacetylation reaction that generates OH37 LPS. Therefore, it is a concern that enzymatically deacetylated LPSs in vivo may facilitate hemoglobin degradation.

Our CD experiments to further describe Hb−LPS interactions identified structural changes consistent with the formation of substantial quantities of metHb, a conclusion similar to that determined from the absorbance spectral analyses. The major CD changes in the visible and Soret regions were consistent with heme oxidation, although the CD spectrum in these regions is also sensitive to the overall quaternary structure of the protein (Sugita et al., 1971; Geraci & Parkhurst, 1981). The lack of CD alteration in the far-UV region of the spectrum (e.g., 210−240 nm) provided evidence that there were no substantial changes in the globin secondary structure (Chen et al., 1972). In general, the overall secondary conformation of Hb is considered to be insensitive to changes in iron valence state and the binding of extrinsic ligands (Myer & Pande, 1978). However, the CD spectrum in the near-UV region (250−300 nm), which also was altered in the presence of LPS, has been suggested previously to be sensitive to the environments of aromatic amino acids at the $\alpha_1\beta_2$ interface (Geraci & Parkhurst, 1981; Zentz et al., 1994) as well as to disulfide chromophores (Zentz et al., 1994), suggesting that there may have been some localized globin conformational changes induced by LPS. Interestingly, there were only minor changes in oxygen affinity associated with LPS binding to Hb. With the conditions of our experiments (i.e., the relative Hb and LPS concentrations and the time of incubation), we previously have demonstrated that Hb and LPS form complexes with approximately equal weights of the two components. Accordingly, each chain of the Hb tetramer might bind a single smooth LPS, or a small number of rough LPSs. On the basis of our previous observations that Hb can interact with rough LPSs and partial lipid A structures (Kaca et al., 1994b) and the known ability of Hb to intercalate into lipid bilayers (Szebeni et al., 1988), it is likely that binding occurs predominantly via interactions between Hb and lipid A. Heme is unlikely to be directly involved in this binding process since the oxygen affinity of Hb is only minimally affected by LPS, although as a result of the binding of LPS there apparently are changes in globin conformation sufficient to destabilize the heme pocket such that the rate of heme oxidation is increased.

LPS-mediated oxidation of Hb to methemoglobin and hemichromes is likely to facilitate iron release and the generation of free radicals, conditions known to contribute to Hb-related toxicity (Sadrzadeh et al., 1984; Simoni et al., 1990; Gutteridge, 1986), although we detected no increase in the production of oxidizing species in Hb/LPS mixtures, compared with Hb alone. We postulate the LPS-induced Hb oxidation may be an important component of Hb toxicity in vivo by a two-step mechanism for Hb toxicity which involves biochemical changes in both LPS and Hb as a result of Hb/LPS complex formation. First, binding of Hb to LPS partially disaggregates the high molecular weight (> 10^6 Da) LPS micelles and enhances LPS biological activity, as we have shown previously (Kaca et al., 1994a,b; Roth et al., 1993; Roth, 1994). Second, Hb/LPS complexes exhibiting high LPS biological activity may then result in a series of secondary reactions which can play important roles in the pathophysiology of endotoxic shock. These reactions include the activation of polymorphonuclear leukocytes with subsequent generation of oxygen free radicals (Yoshikawa, 1990), the enhanced production of procoagulant activity from both mononuclear cells (Roth et al., 1993) and endothelial cells (Roth, 1994), and the facilitation of Hb oxidation with heme or iron release and production of oxygen free radicals (Gutteridge, 1986). The formation of hydroxyl radicals can cause activation of the complement cascade (Shingu et al., 1989, 1992) and other harmful reactions, leading to organ dysfunction, that have previously been described following the administration of LPS-containing or stromal lipid-containing hemoglobin solutions (Feola et al., 1988a).

In conclusion, by analysis of absorption and circular dichroic spectra of Hb, we have shown that enterobacterial endotoxins can facilitate degradation of the Hb molecule through mechanisms that potentially involve heme loss, increased rate of iron oxidation, and conversion of Hb to hemichromes and methemoglobin. All of these changes are along the pathway to Hb denaturation and may contribute to in vivo toxicity of cell-free hemoglobin. On the basis of an approximately equal weight ratio of Hb and LPS in Hb/LPS complexes and the typical LPS plasma concentrations encountered during endotoxemia (picograms to nanograms per milliliter), only a small fraction of infused Hb would be expected to initially undergo LPS-facilitated degradation. However, endotoxemia can persist during sepsis despite detoxification mechanisms that normally result in rapid LPS clearance, indicating continuous influx into the circulation of new LPS from an infectious focus or the gastrointestinal tract. The continuous or recurrent entry of LPS into the circulating blood would be expected to result in an increasing fraction of denatured Hb during the prolonged circulation of the infused Hb.

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Activation of complement by human hemoglobin and by mixtures of hemoglobin and bacterial endotoxin

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Abstract

Purified human hemoglobin is being developed as an alternative to transfusions of homologous erythrocytes. However, toxicity associated with infusion of hemoglobin has limited the development of this resuscitation fluid. Some observed toxicities, including activation of the complement cascade, have been associated with contamination of hemoglobin solutions by bacterial endotoxin. Recent studies have demonstrated complex formation between hemoglobin and endotoxin, and have documented a resultant increase in the ability of endotoxin to activate coagulation, stimulate tissue factor production by human peripheral blood mononuclear cells, and stimulate tissue factor activity and protein synthesis in cultured human endothelial cells. The process of hemoglobin enhancement of endotoxin toxicity suggests a possible mechanism by which the consequences of endotoxin contamination of hemoglobin solutions, including complement activation, could be magnified. Therefore, we studied the potential of hemoglobin to either fix complement directly, or modify the ability of endotoxin to fix complement. Human crosslinked and native hemoglobins, at concentrations between 0.2 mg/ml and 3 mg/ml, were shown to fix complement. Complement fixation by hemoglobin was identical in normal human serum or in factor B-depleted serum, suggesting that fixation occurred via the classical pathway of complement activation. Complement fixation then was examined with a battery of smooth and rough endotoxins tested in the absence and presence of hemoglobin. Addition of hemoglobin to a solution of a rough Salmonella endotoxin partial structure, from which a single fatty acid had been hydrolyzed from the lipid A portion of the macromolecule, resulted in decreased efficiency of complement fixation. However, addition of hemoglobin had little or no effect on the intrinsic complement fixing abilities of eight other smooth endotoxins, rough endotoxins, or endotoxin partial structures. Our results demonstrated the ability of hemoglobin to fix complement at hemoglobin concentrations which would be achieved during infusion for resuscitation, but failed to demonstrate a reproducible effect of hemoglobin on the activation of complement by endotoxin.

Keywords: Complement activation; Cell-free hemoglobin; Crosslinked hemoglobin; Bacterial lipopolysaccharide

1. Introduction

The development of blood substitutes as an alternative to donated erythrocytes is of great importance in both military and civilian settings. During the past two decades, solutions of unmodified hemoglobin (HbA_0), crosslinked, conjugated or polymerized Hb, and encapsulated Hb have been prepared and evaluated for efficacy in resuscitation models. Modified Hbs are of particular interest because their in vivo half-lives are prolonged compared to native HbA_0. Many preparations of modified Hb also have been demonstrated to retain adequate properties in regard to oxygen binding and delivery, low viscosity, lack of infectious risk, and in vitro stability for long-term storage [1–3]. However, the clinical development of crosslinked Hb and other hemoglobin-based blood substitutes has been slow primarily because of systemic toxicities, and presently the principal limitation for the utilization of Hb for human use is in vivo toxicity. Infusions of Hb into humans and animals have resulted in fever, hypertension, thrombocy-

Abbreviations: Hb, cell-free hemoglobin; $\alpha\alpha$Hb, bis(3,5-dibromosalicyl)fumarate $\alpha\alpha$-crosslinked cell-free hemoglobin; HbA_0, native hemoglobin A_0; LPS, bacterial lipopolysaccharide.

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topenia, activation of proteolytic cascades, disseminated intravascular coagulation with parenchymal organ damage, reduced tolerance to sepsis, susceptibility to bacterial infections, reticulo-endothelial cell blockade and lethal toxicity [4–13]. In vitro, Hb has been shown to stimulate tissue factor production by mononuclear cells [14,15] and cause endothelial cell injury [16].

One poorly understood mechanism for some aspects of Hb toxicity is complement activation. Some preparations of cell-free Hb have resulted in complement activation in vitro and in vivo [6–8,12,17–20]. In some studies, complement activation was observed with Hb preparations that contained stromal phospholipid and/or bacterial endotoxin (lipopolysaccharide, LPS), but not with Hb preparations without detectable levels of these contaminants [7,8,18,19,21]. It was suggested that these contaminants, and not Hb, might be responsible for complement activation. Complement binding and activation by LPS is a well-understood process [22–24], and this is an attractive mechanism for the complement activation that has been observed with some preparations of Hb. In addition, recent studies have demonstrated that biological processes initiated by LPS may be modified by Hb. In particular, it has been recently recognized that Hb forms stable complexes with LPS [25], and that in the presence of Hb there is in vitro enhancement of many important biological activities of LPS, including activation of coagulation proteases [26,27], mononuclear cells [15], and endothelial cells [28]. In vivo, it has been demonstrated that co-infusion of Hb and LPS results in synergistic toxicity (e.g., lethality) compared to the toxicity of LPS or Hb alone [29].

Based on the known interaction between Hb and LPS, we have investigated the possibility that Hb may modify the ability of LPS to bind and activate complement. LPS is a ubiquitous environmental contaminant that is frequently introduced during the preparation of Hb; i.e., LPS can contaminate the preparations of starting erythrocytes, solutions and equipment used for biochemical purification of Hb, solutions used for chemical modification of Hb, and materials used for formulation of the final product for in vivo use. Even low levels of LPS contamination become a major clinical concern when large volumes of Hb solution are required for infusion. Alternatively, there is the possibility that the infusion of Hb may potentiate the toxicity of pre-existing endotoxemia. Physiologically significant levels of LPS are present in the circulating blood in a variety of clinical conditions, including sepsis, hepatic injury, hypotension, and damage to the gastrointestinal tract. Since many clinical circumstances for which Hb would be administered are likely to be associated with shock and hypoxia (pathological states that lead to deterioration of mucosal barriers and hepatic function), significant quantities of endotoxin would be expected to be present in the circulation of many patients receiving Hb. The synergistic toxicity produced by Hb and LPS thus could potentially magnify the deleterious effects of endotoxemia even if the infused Hb was 'endotoxin-free'. The aim of our study was to determine whether Hb could modulate the ability of LPS to activate complement.

2. Materials and methods

Lyophilized human serum, obtained from Diamedix (Miami, FL), was reconstituted in endotoxin-free water and stored at −70°C. Human factor B depleted serum was purchased from Quidel (San Diego, CA). Human immune gamma globulin (IgG) for intramuscular injection (185 mg/ml) was purchased from Armour Pharmaceutical (Kankakee, IL), and human serum albumin (HSA) (25%, for injection) was purchased from Nybcen (New York, NY). Sheep erythrocytes, sensitized with IgG, were obtained from Diamedix (Miami, FL).

Human hemoglobin (Hb) was prepared and purified, as described previously [30], by collaborators at the Blood Research Division of the Letterman Army Institute of Research (BRD/LAIR), San Francisco, CA. The Hb was covalently crosslinked between α chains with bis(3,5-dibromosalicyl)fumarate ($\alpha\alpha$Hb), and at a final concentration of 8.4 g/dl contained less than 0.4 EU/ml endotoxin (referenced to *E. coli* lipopolysaccharide B, O55:B5, Difco Laboratories, Detroit, MI) as determined by the Limulus amebocyte lysate (LAL) test [31]. The Hb did not contain detectable stroma based on analysis for phosphorus. Purified native non-crosslinked A_o (HbA_o), 8.4 g/dl, was prepared from Hb by ion exchange HPLC, as described previously [32]. Methemoglobin (met$\alpha\alpha$Hb) was prepared by oxidation of $\alpha\alpha$Hb with potassium ferricyanide at 4°C in the dark for 30 min with occasional mixing [33]. The molar ratio of $K_3Fe(CN)_3$ to $\alpha\alpha$Hb was 1.2:1. Met$\alpha\alpha$Hb was separated from ferrocyanide and residual ferricyanide by Sephadex G-25 M (PD-10, Pharmacia, Piscataway, NJ) column chromatography, and the methemoglobin stock solution (3 mg/ml) was stored at 4°C in 0.05 M potassium phosphate (pH 7.4).

2.1. Bacterial endotoxin (lipopolysaccharide, LPS)

Salmonella minnesota Re 595, *Proteus mirabilis* S1959, 03, R110 and R45 LPSs, and F203, the lipid A-free core oligosaccharide of *P. mirabilis* 03 LPS, were prepared as described previously [34–37]. *S. minnesota* 595 LPS was chemically modified to produce singly deacylated OH37 LPS (lacking one fatty acid from the lipid A portion of the macromolecule), multiply deacylated OH56 LPS (lacking several fatty acids from lipid A), lipid A, and monophosphoryl lipid A [38,39]. The Proteus and Salmonella LPSs were provided by collaborators at the Institute of Microbiology and Immunology, University of Lodz, Poland. Other biological activities and structural characteristics of these Proteus and Salmonella LPSs related to their potential for interaction with Hb have recently been described

(Kaca, W., Roth, R.I., Vandegriff, K., Chen, G.C., Kuypers, F.A. and Levin, J., unpublished data) [27].

2.2. Complement assay

Complement fixation was determined in V-bottomed microtiter plates (Becton-Dickinson, Lincoln Park, NJ) by a modification of the procedure described by the manufacturer of the sensitized sheep erythrocytes (Diamedix). Serial dilutions of proteins, LPSs or mixtures of both were prepared in veronal buffer, pH 7.4, containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$, and 50 μl aliquots were incubated for 30 min at 37°C. 25 μl of serum, typically diluted 1:10 or 1:15 in veronal buffer (dilutions sufficient to produce 60–80% lysis of erythrocytes), then was added and the mixtures incubated an additional 60 min at 37°C. 150 μl of sensitized sheep erythrocytes then were added and incubated 60 min at 37°C with gentle mixing, after which the unlysed erythrocytes were pelleted by centrifugation at $3000 \times g$ for 15 min at room temperature in a Sorvall Technospin R centrifuge (DuPont, Wilmington, DE). 150 μl of the supernatants then were transferred to flat bottom Falcon microtest II culture plates (Becton Dickinson, Lincoln Park, NJ) and absorbances were measured at 405 nm in a plate reader (Kinetic-QLC, Whittaker Bioproducts, Walkersville, MD) in order to quantify free Hb produced by erythrocyte lysis. Background absorbance at 405 nm (which in some samples included a component due to Hb) was subtracted from each reading. In some experiments, the measured absorbances are expressed as residual hemolytic activity by comparison with the absorbance at 405 nm generated by lysis of all erythrocytes. 2–8 replicate wells were run for each experimental sample, and each experiment was repeated at least two times. Representative results are presented.

3. Results

3.1. Complement fixation by Hb and other human proteins

Native HbA_0, $\alpha\alpha$-crosslinked Hb, HSA and IgG were each tested for ability to fix human complement. In a representative experiment, both Hb preparations fixed complement at high protein concentrations (threshold at 2.5 mg/ml) (Fig. 1). IgG fixed complement at lower protein concentrations (< 1 mg/ml), whereas HSA did not fix complement at any protein concentration tested (Fig. 1). Threshold Hb concentrations for complement fixation ranged from 0.2–3 mg/ml in nine independent experiments, and calculated EC_{50} values ranged from 1.2–2.6 mg/ml. In a control experiment to rule out the possibility that Hb had altered the erythrocytes so that they were no longer susceptible to complement lysis, erythrocytes were pre-treated with a high concentration of $\alpha\alpha$Hb (1 mg/ml, a concentration sufficient in this experiment to result in little or no residual complement activity in the incubation buffer), and the erythrocytes subsequently re-purified by centrifugation, washed, and subjected to lysis with complement. Lysis of these erythrocytes was normal, indicating that the potential binding of $\alpha\alpha$Hb to the erythrocytes did not affect the subsequent ability of these cells to measure residual complement activity. Complement fixation by $\alpha\alpha$Hb was shown to be identical in normal human serum or factor B (alternative pathway) depleted serum (Fig. 2), indicating that fixation occurred via the classical pathway

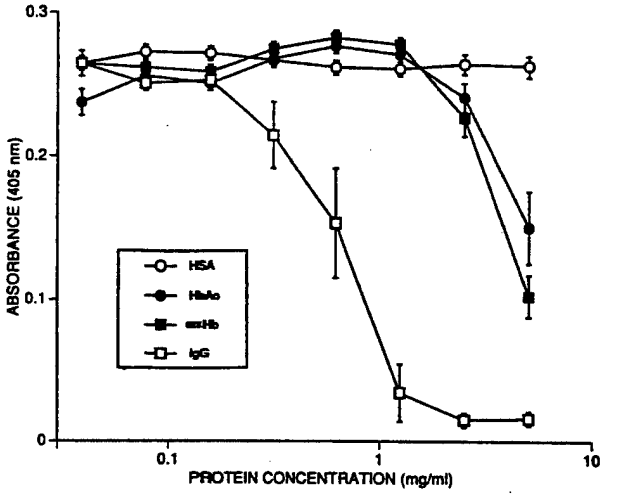

Fig. 1. Complement fixation in the presence of human proteins. Serial dilutions of human serum albumin (HSA), purified native human hemoglobin (HbA_0), cell-free crosslinked human hemoglobin ($\alpha\alpha$Hb) and human immunoglobulin (IgG) were incubated with human serum, and residual complement activity (measured at 405 nm) was determined as described in Materials and methods. Samples were run in duplicate, and mean values with ranges are presented.

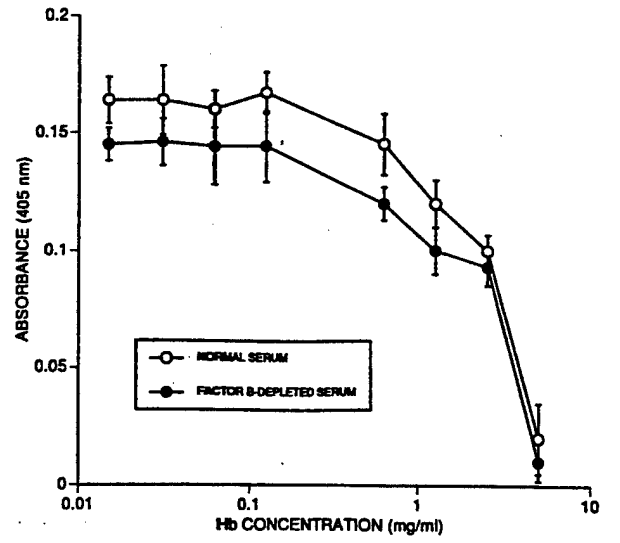

Fig. 2. Complement fixation in the presence of Hb: effect of factor B-depleted serum. Serial dilutions of crosslinked human hemoglobin ($\alpha\alpha$Hb) were incubated with complete human serum or factor B-depleted serum, and residual complement activity (measured at 405 nm) was determined as described in Materials and methods. Samples were run in duplicate, and mean values with ranges are presented.

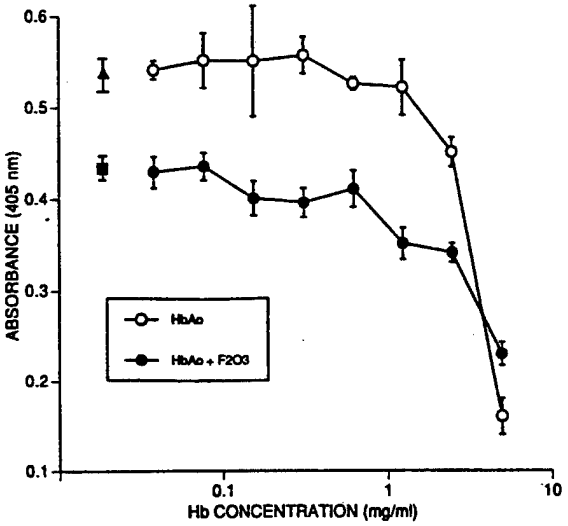

Fig. 3. Complement fixation in the presence of Hb: effect of core oligosaccharide. Serial dilutions of native hemoglobin (HbA_o) were incubated with complete human serum in the presence or absence of 20 μg/ml *P. mirabilis* O3 core oligosaccharide (F2O3), and residual complement activity (measured at 405 nm) was determined as described in Materials and methods. Initial complement activity of serum (▲) and residual complement activity after incubation with F203 in the absence of Hb (■) are shown. Samples were run in duplicate, and mean values with ranges are presented.

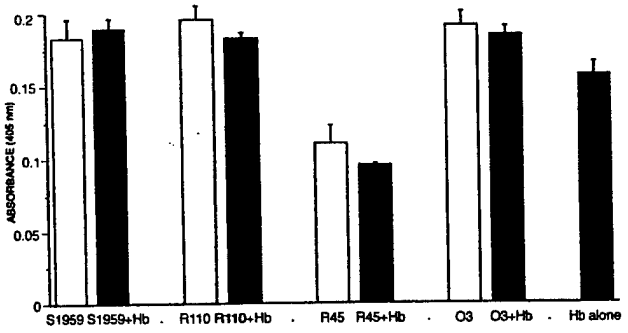

Fig. 4. Effect of Hb on complement fixation by smooth and rough Proteus LPSs. Smooth *P. mirabilis* S1959 and 03 LPSs, and rough R110 and deep rough R45 LPSs (each at 25 μg/ml), in the absence (closed bar) or presence (open bar) of crosslinked human hemoglobin ($\alpha\alpha$Hb, 0.5 mg/ml), were incubated with complete human serum, and residual complement activity was determined as described in Materials and methods. Mean values ± 1 S.D. of eight replicate samples are presented.

of complement activation. Further evidence that the alternative pathway of complement activation was not involved in the interaction with Hb was provided by the finding that HbA_0, in concentrations as high as 1 mg/ml, did not interfere with the ability of isolated *P. mirabilis* 03 core oligosaccharide (F203) (20 μg/ml) to fix complement via the alternative pathway (Fig. 3). ANOVA analysis between groups (HbA_0 alone vs. HbA_0 + F203) demonstrated differences ($P < 0.05$) for each of the Hb concentrations below 5 mg/ml except at 0.16 mg/ml ($P = 0.14$). Finally, fixation of complement by met$\alpha\alpha$Hb was contrasted with that by $\alpha\alpha$Hb, each at 1.0 mg/ml. Fixation by met$\alpha\alpha$Hb compared to $\alpha\alpha$Hb was 20% vs. 64% and 15% vs. 38%, respectively, in two independent experiments (data not shown).

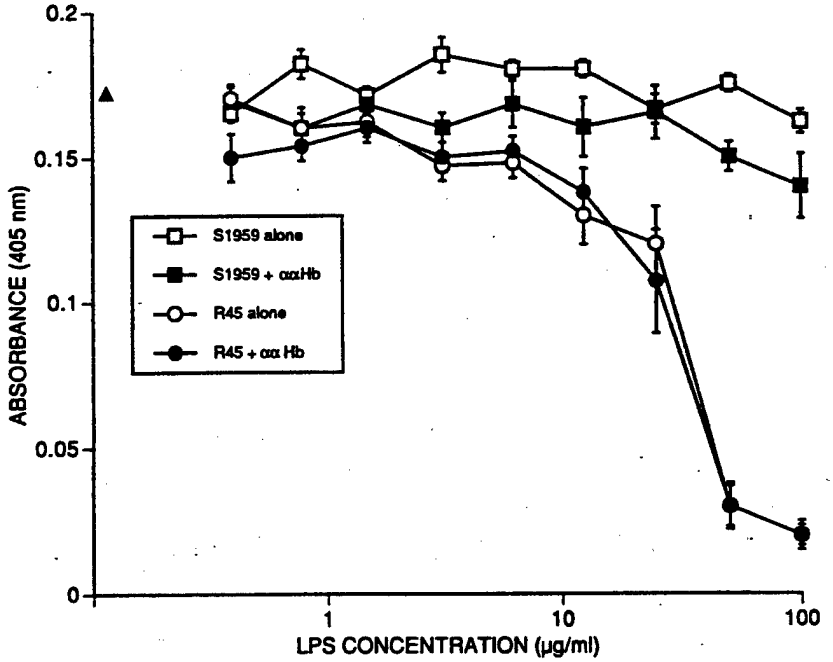

Fig. 5. Effect of Hb on complement fixation by smooth and rough Proteus LPSs. Serial dilutions of smooth *P. mirabilis* S1959 and deep rough R45 LPSs, in the absence or presence of crosslinked human hemoglobin ($\alpha\alpha$Hb, 0.5 mg/ml), were incubated with complete human serum, and residual complement activity was determined as described in Materials and methods. Complete erythrocyte lysis (▲) is shown. Mean values ± 1 S.D. of eight replicate samples are presented.

3.2. Influence of Hb on complement fixation by LPSs

A large series of LPSs, with various intrinsic abilities to fix complement, were studied in order to assess the potential of Hb to modify their effectiveness in binding complement. Two smooth Proteus LPSs (S1959 and 03) and a rough (Ra type) Proteus LPS containing a truncated polysaccharide portion (R110), each at 25 μg/ml, demonstrated little complement fixing ability in either the absence or presence of 0.5 mg/ml $\alpha\alpha$Hb (Fig. 4), a concentration of Hb insufficient in this experiment to fix detectable quantities of complement by itself. ANOVA analysis demonstrated no differences within or between these groups. A deep rough (Re type) Proteus LPS (R45), containing only lipid A, Kdo, and 4-amino-arabinose residues, was capable of fixing complement (50% complement fixation at 25 μg/ml LPS; $P < 0.01$ for comparison with the parent S1959 LPS), but also demonstrated little change in

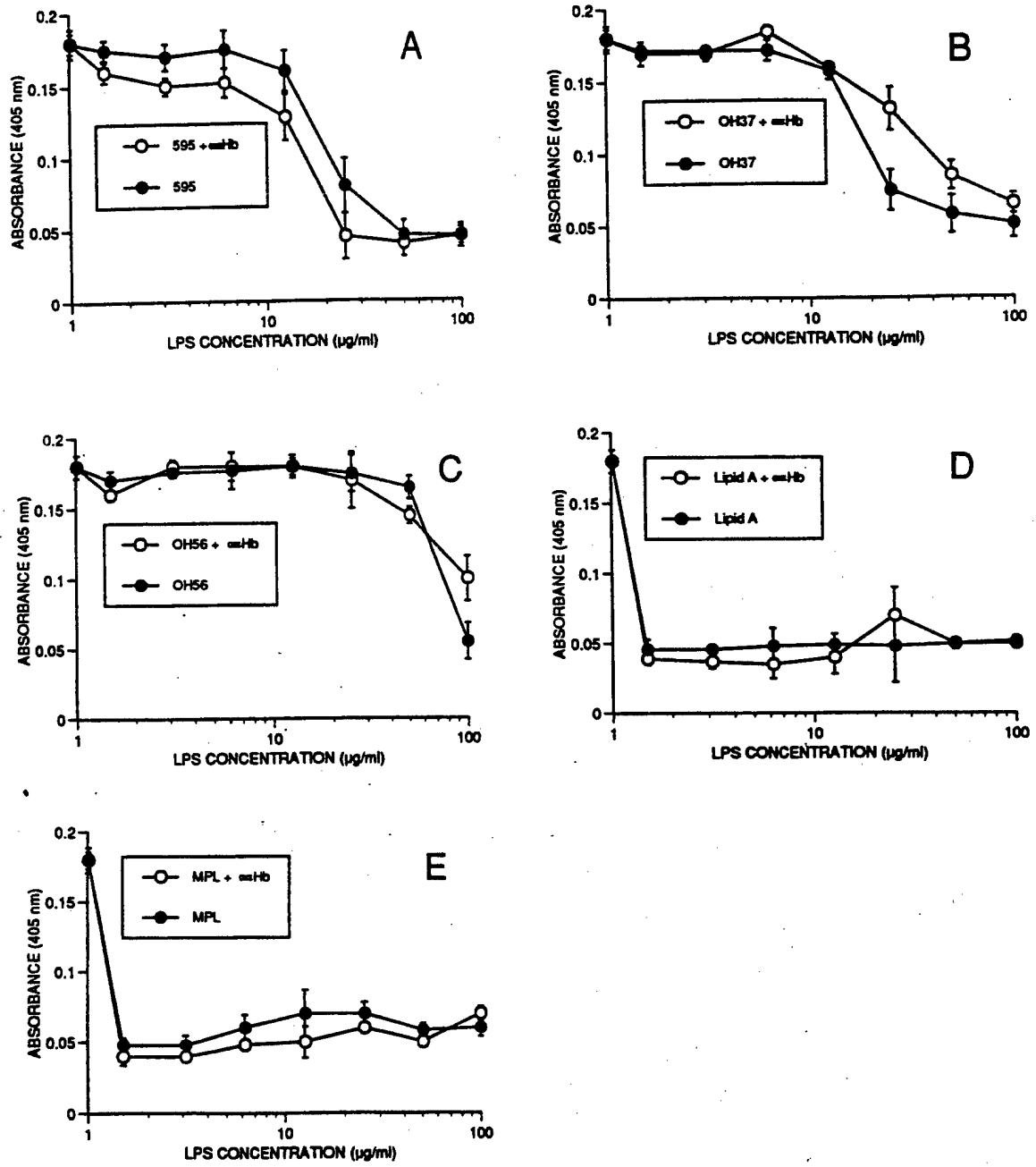

Fig. 6. Effect of Hb on complement fixation by Salmonella LPSs. Serial dilutions of deep rough *S. minnesota* 595 LPS, singly deacylated 595 LPS (OH37), lipid A, multiply deacylated 595 LPS (OH56), and monophosphoryl lipid A (MPL), in the absence (●) or presence (○) of crosslinked human hemoglobin ($\alpha\alpha$Hb, 0.5 mg/ml), were incubated with complete human serum and residual complement activity was determined as described in Materials and methods. Mean values ± 1 S.D. of eight replicate samples are presented.

the presence of 0.5 mg/ml $\alpha\alpha$Hb ($P = 0.1$) (Fig. 4). There was also no effect on complement fixation by these LPSs in the presence of non-crosslinked HbA_0. In order to ensure that the lack of an observable Hb effect was not due to inappropriate incubation conditions (e.g., relative LPS and Hb concentrations that might be insensitive to the potential effect of Hb), one of the smooth LPSs (S1959) and the rough R45 LPS were further investigated for complement fixation, in the absence or presence of $\alpha\alpha$Hb, over a wide range of LPS concentrations. $\alpha\alpha$Hb (0.5 mg/ml) did not alter either the ability of R45 LPS, or the inability of S1959 LPS, to fix complement at LPS concentrations ranging from 0.5 μg/ml to 100 μg/ml (Fig. 5).

The availability of several chemically modified, partial structures of another rough LPS, *Salmonella minnesota* Re 595, made possible a more detailed investigation of the potential of Hb to modify complement fixation by the lipid A portion of the LPS macromolecule (i.e., by the classical pathway of complement activation). In the absence of Hb, native *S. minnesota* 595 LPS and singly deacylated 595 LPS (OH37) demonstrated identical LPS concentration-dependent complement fixation (Fig. 6A and B), whereas multiply deacylated 595 LPS (OH56) was much less biologically active (Fig. 6C), and lipid A alone or monophosphoryl lipid A were much more active (Fig. 6D and E). In the presence of 0.5 mg/ml $\alpha\alpha$Hb, a modest decrease in the ability of OH37 to fix complement was observed at 25 μg/ml OH37 ($P = 0.002$) and 50 μg/ml OH37 ($P = 0.03$), and the concentration of OH37 LPS required to produce 50% inhibition of complement activity was increased from 15 μg/ml to 38 μg/ml (Fig. 6B). The other LPSs tested were not appreciably affected by $\alpha\alpha$Hb. Therefore, with the sole exception of OH37 LPS, the various abilities of a battery of smooth and rough LPSs, and rough LPS partial structures, to fix complement were unchanged by Hb.

4. Discussion

Cell-free Hb and LPS can co-exist in the bloodstream by two mechanisms: firstly, infusions of Hb for resuscitation could be contaminated with LPS or could be administered during in vivo endotoxemia associated with the underlying clinical condition (e.g., trauma requiring transfusion); or secondly, cell-free Hb could interact with LPS following the destruction of erythrocytes by bacterial hemolysins [40,41] or by mechanical hemolysis during disseminated intravascular coagulation [42,43]. Complement activation is a prominent pathophysiologic component of endotoxemia [44], and the ability of Hb to modulate complement activation is a potentially important clinical aspect of hemoglobinemia. Therefore, our studies were undertaken to investigate the potential of Hb to fix complement directly or, alternatively, to influence the ability of bacterial endotoxins to fix complement.

Our results demonstrated that hemoglobin, at threshold concentrations between 0.2 mg/ml and 3 mg/ml in many experiments, fixed complement independently of LPS. This effect was demonstrated for both native and $\alpha\alpha$-crosslinked Hbs, indicating that this biochemical interaction was a basic property of Hb and that the mechanism of complement binding to Hb did not involve dissociation of the chains of the Hb tetramer. The finding that Hb efficiently fixed complement from factor B-depleted serum suggested that this process involved the binding of a factor(s) of the classical pathway of complement activation. The observation that Hb did not interfere with complement fixation by isolated core saccharide (which is known to activate complement by the alternative pathway [23]) provided further support for the conclusion that Hb interacts with the classical complement pathway. It is possible that the mechanism of the binding of complement to Hb is similar to that of complement activation by serum mannan-binding protein, which initiates the complement cascade by replacing C1q molecules [45], or by two major outer membrane proteins from Klebsiella pneumoniae, which initiate the cascade by binding C1q [46]. The concentrated Hb stock solutions utilized for our studies had less than 0.4 EU/ml LPS, as determined by the LAL test, and had no detectable stroma. At the concentrations of Hb utilized for our studies (≤ 5 mg/ml), LPS contamination was ≤ 0.01 EU/ml, a concentration several thousand-fold lower than that required to activate complement in our hemolytic assay. Therefore, complement fixation was shown to be a property of Hb rather than that of LPS contamination. In a previous study demonstrating activation of the alternative pathway of complement by Hb [17], LPS contamination was not evaluated. It is not clear whether the failure of several other previous studies [6,8,18,19] to detect complement activation by Hb, in contrast to our reproducible detection of this process, reflect differences in the Hb preparations studied or rather differences in the complement assays utilized to search for a Hb-complement interaction. It is possible that chemical contaminants associated with filtration steps in the preparation of Hb may contribute to complement fixation [47].

The failure of Hb to reproducibly alter complement fixation by LPS was surprising. LPS has the ability to activate complement by both the classical pathway, via an interaction between C1q, antibody and the lipid A moiety of LPS [23,24,48] or by the alternative pathway, via an interaction between complement factors C3, B and H and the core and polysaccharide components of LPS [22]. Extensive studies in our laboratory have previously demonstrated the ability of Hb to bind and dissociate LPS [25,26], and subsequently augment several biological activities of LPS, including LPS stimulation of coagulation [25–27], mononuclear cell tissue factor production [15,26], and endothelial cell tissue factor production [26–28]. The increased LPS biological activity that accompanied Hb binding was associated with dissagregation of high molec-

ular weight LPS particles, and it was hypothesized that Hb increased the availability of lipid A, the biologically active portion of the LPS macromolecule. Therefore, it seemed possible that complement fixation could also be enhanced by Hb. However, a battery of smooth and rough LPSs, including LPS partial structures otherwise shown to have biological activity [27], were generally unaffected by Hb in regard to their intrinsic abilities to fix complement. Only with singly deacylated *S. minnesota* OH37 LPS was a Hb effect noted; i.e., the complement activating potency of OH37 was decreased at 25 and 50 μg/ml LPS after interacting with Hb ($P < 0.05$ for each comparison by ANOVA analysis). Interestingly, OH37 LPS also was found to be the most effective of the chemically modified *S. minnesota* LPSs tested in induction of oxidative denaturation of Hb, with subsequent production of metHb and hemichromes (Kaca, W., Roth, R.I., Vandegriff, K., Chen, G.C., Kuypers, F.A. and Levin, J., unpublished data). This suggests that production of oxidized Hb by LPS interferes with complement fixation, a possibility supported by our finding that metHb fixed complement much less efficiently than did Hb.

The abilities of specific LPS partial structures to fix complement were not appreciably altered by the presence of Hb. Comparison of the complement activating capabilities of the chemically modified Salmonella Re LPSs, in both the absence and presence of Hb, demonstrated that multiply deacylated OH56 LPS lost much of its complement fixing activity compared to the naturally occurring 595 LPS ($P < 0.05$ by ANOVA analyses for each of the comparison of groups at 25, 50 and 100 μg/ml LPS). This finding confirms the previously noted importance of ester-bound fatty acids for complement binding [49]. The lesser efficiency of complement activation by the parent 595 LPS, compared to lipid A which was produced by hydrolysis of the Kdo residues of 595 LPS, also confirms the previously recognized ability of the saccharide components of LPS to block classical pathway complement activation by lipid A [23,24,48]. Since the presence of 0.5 mg/ml Hb (a concentration sufficient by itself to bind measurable quantities of complement in most experiments) did not alter complement fixing activity of 0.001–0.1 mg/ml of most of the LPSs tested, it is likely that the affinity of complement proteins for LPS is substantially greater than the affinity of complement proteins for Hb.

The observation that our battery of smooth LPSs, rough LPSs and partial LPS structures were generally unaltered in complement fixing abilities in the presence of Hb is very different from the results of similar experiments performed to test the influence of Hb on the ability of LPS to activate coagulation. This well-known LPS biological activity was dramatically enhanced by Hb when complete smooth or rough LPSs were tested, but was generally unaffected by Hb when lipid A or LPS partial structures were tested, suggesting a role for the saccharide components of LPS in the Hb enhancement effect [27]. Therefore, it is possible that the difference between the effects of Hb on the LPS-mediated activation of the coagulation and complement protease cascades is attributable to a different presentation to the proteases of critical LPS chemical structures.

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Hemoglobin, a Newly Recognized Lipopolysaccharide (LPS)-binding Protein That Enhances LPS Biological Activity*

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Cell-free hemoglobin (Hb) is a purified preparation of human hemoglobin that is being developed as a resuscitation fluid. *In vivo* administration of hemoglobin has resulted in significant toxicity, due in part to contamination with bacterial endotoxin (lipopolysaccharide (LPS)). To better understand this toxicity, we have studied the interaction between Hb and LPS. Mixtures of each of three different Hb preparations (cross-linked αα-Hb, cross-linked carbon monoxy-αα-HbCO, and noncross-linked (native) HbA_0) and LPS (*Escherichia coli* O26:B6 or *Proteus mirabilis* S1959) were examined by several independent methods for evidence of Hb·LPS complex formation. Binding assays in microtiter plates demonstrated saturable binding of LPS to immobilized Hb, with a k_D of 3.1×10^{-8} M. Binding of LPS to Hb also was demonstrated with a radiolabeled LPS photoaffinity probe. Ultrafiltration of Hb/LPS mixtures by 300- and 100-kDa cut-off membranes showed that the majority of LPS in these mixtures (87–97 and 64–72%, respectively) was detected in the filtrates, in contrast to the lack of filterability of LPS in the absence of Hb. Density centrifugation demonstrated that LPS co-migrated with each of the three Hbs, whereas unbound LPS had a distinctly greater sedimentation velocity than Hb or Hb·LPS complexes. Nondenaturing polyacrylamide gel electrophoresis demonstrated that in the presence of Hb, LPS migrated into the gel and co-electrophoresed with Hb, whereas LPS alone did not appreciably enter the gel. Finally, precipitation by ethanol of each of the three Hb preparations was increased in the presence of LPS compared with precipitation in the absence of LPS. Interaction of LPS with each of the three Hb preparations was also associated with altered biological activity of LPS, as shown by enhancement of LPS activation of *Limulus* amebocyte lysate. Therefore, our data provide several lines of independent evidence for Hb·LPS complex formation and indicated that LPS exhibited altered physical characteristics and enhanced biological activity in the presence of Hb.

Cell-free hemoglobin (Hb)[1] is a preparation of human hemoglobin that is being developed for use as an oxygen-transporting resuscitation fluid (1, 2). Hb has excellent oxygen delivery properties and a long shelf life and, therefore, is a potentially ideal red blood cell substitute. However, Hb has not yet been used clinically because of significant problems of toxicity. Hypertension and bradycardia have been commonly observed (3, 4), and a decrease in glomerular filtration rate and renal plasma flow have been described (5). Mild prolongations of the partial thromboplastin time also have been reported (4). In some animal studies, preparations of Hb have been shown to produce fever, disseminated intravascular coagulation with resultant thrombosis, and ischemic parenchymal damage (6, 7).

Whether the reported toxicity is due to hemoglobin, *per se*, or to contaminants such as stromal phospholipids or bacterial endotoxin (lipopolysaccharide, LPS) is still uncertain, and inconsistent results have been described. Widespread parenchymal organ damage and activation of the complement and coagulation cascades have been demonstrated in hemoglobin preparations that contained detectable stromal phospholipids (6–8). Similarly, increased lethality in rabbits that received Hb contaminated with LPS, compared with Hb in the absence of detectable LPS, has indicated a role for endotoxin in causing *in vivo* toxicity of Hb (9, 10). In contrast, hepatotoxicity has been reported in the absence of detectable LPS or stromal lipid (7), thus suggesting intrinsic hemoglobin toxicity.

Because it remains unknown whether Hb binds LPS, and since binding could alter the biological activity of LPS, the present study was designed to evaluate the interaction between these molecules. Our data indicate that complex formation occurs between Hb and LPS, and that the procoagulant activity of Hb·LPS is increased compared with LPS alone.

MATERIALS AND METHODS

Reagents—Falcon centrifuge tubes (sterile, 15 ml) were obtained from Becton Dickinson (Mountainview, CA). Sterile, endotoxin-free water and 0.9% NaCl were purchased from Travenol Laboratories (Deerfield, IL). RNase and DNase were purchased from Sigma.

Glassware—All glassware was rendered endotoxin free by heating at 190 °C in a dry oven for 4 h.

Hemoglobin—Human cell-free hemoglobin (Hb), prepared and purified as described previously (11, 12), was provided by collaborators at the Blood Research Division of the Letterman Army Institute of Research, San Francisco, CA. Hb cross-linked between α chains with bis(3,5-dibromosalicyl)fumarate (αα-Hb) was 9.6 g/dl (95.4% cross-linked, 96.3% oxyhemoglobin, 3.2% methemoglobin), pH 7.4, in Ringers acetate and contained less than 0.4 endotoxin units/ml (referenced to *Escherichia coli* lipopolysaccharide B, O55:B5, Difco), as determined by the *Limulus* amebocyte lysate (LAL) test (13). The αα-Hb stock solution was stored at −70 °C. Carbon monoxyhemoglobin (αα-HbCO) was produced by incubation of the αα-Hb solution with CO and also was at 9.6 g/dl (95.4% cross-linked, 95% HbCO, and 5% oxyhemoglobin). Purified noncross-linked hemoglobin A_0 (HbA_0), 8.4 g/dl, was prepared as described previously (14).

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[1] The abbreviations used are: Hb, cell-free human hemoglobin; αα-Hb, bis(3,5-dibromosalicyl)fumarate αα-cross-linked cell-free hemoglobin; αα-HbCO, αα-cross-linked cell-free carbonmonoxyhemoglobin; HbA_0, noncross-linked cell-free hemoglobin A_0; LPS, bacterial lipopolysaccharide; LAL, *Limulus* amebocyte lysate; HSA, human serum albumin; PBS, phosphate-buffered saline.

Albumin—Human serum albumin (HSA) 25%, for injection) was purchased from Nybcen (New York, NY).

Endotoxins—*E. coli* O26:B6 (W) and O55:B5 (B) LPS were purchased from Difco. Lipopolysaccharide from *Proteus mirabilis* S1959, purified by sequential treatment with RNase and DNase followed by ultracentrifugation (15, 16), has been described previously (16–18) and was provided by collaborators at the Institute of Microbiology and Immunology, University of Lodz, Poland. ^{14}C-Lipopolysaccharide (*Salmonella typhimurium* PR122(Rc), 1 μCi/mg) was purchased from List Biologicals, Inc. (Campbell, CA). ^{125}I-Lipopolysaccharide (*E. coli* O26:B6, 0.1 μCi/μg) was prepared as described previously (19). *Salmonella minnesota* 595 LPS, Re type, extracted by the phenol/chloroform/petroleum ether method (20), was utilized to prepare the photoaffinity probe ^{125}I-LPS-ASD (*S. minnesota* Re595 LPS-(*p*-azidosalicylamido)-1,3′-dithiopropionamide) as described previously (21).

Limulus Amebocyte Lysate—Amebocyte lysates were prepared from *Limulus polyphemus* (the North American horseshoe crab) by disruption of washed amebocytes in distilled water, as described previously (13, 22).

Chromogenic Substrate—Chromogenic substrate S-2423 (AB Kabi Vitrum, Molndal, Sweden) was the gift of Dr. Petter Friberger and was reconstituted with pyrogen-free water.

Chromogenic LAL Test—Activation of LAL by endotoxin was used 1) to compare the biological activity of LPS in the presence and absence of Hb and 2) to determine LPS concentrations in samples after filtration procedures. Dilutions of endotoxins or endotoxin-containing protein solutions were prepared, using pyrogen-free 0.9% NaCl, in sterile, 96-well, flat bottom Falcon microtest II tissue culture plates (Becton Dickinson, Mountain View, CA). 50 μl of sample and 30 μl of LAL (freshly diluted 1:20 in 0.9% NaCl prior to use) were incubated in tissue culture plates for 30 min at 37 °C in a temperature-controlled plate reader (Kinetic-QCL, Whittaker Bioproducts Inc., Walkersville, MD). 40 μl of chromogenic substrate S-2423 (0.25 mM, in 25 mM Tris, pH 8.6) was then added to each well. Mixtures were incubated at 37 °C for 5 min, and absorbances at 405 nm were determined. Background absorbance at 405 nm (which included a component of absorbance due to Hb) was subtracted from each reading. Samples were assayed in duplicate or triplicate.

Gelation LAL Test—Samples were assayed for Hb or HSA enhancement of the biological activity of LPS with the LAL test using gelation as the end point (13, 22). LPS concentrations in samples were calculated based on a LPS standard curve established with *E. coli* O55: B5.

Binding of LPS to Hb-coated Microtiter Plate Wells—Incubations of LPS and Hb in microtiter plates were utilized to demonstrate binding of LPS to immobilized Hb and determine affinity. αα Hb (1 μg/well in phosphate-buffered saline, pH 7.4 (PBS)) was added to each well of a 96-well polyvinyl soft round bottomed microtiter plate and incubated at 37 °C overnight. Wells were then washed 3 times with PBS, and excess binding sites were blocked with 100 μl of bovine serum albumin/well (1 mg/ml). After 2 h, unbound bovine serum albumin was removed with three PBS washes, and 100 μl of various concentrations of ^{125}I-LPS (*E. coli* O26:B6 LPS, 1.7×10^4 cpm/μg) in PBS was added. In control experiments to determine nonspecific binding, ^{125}I-LPS was added to bovine serum albumin-blocked wells in the absence of Hb. Following a 4-h incubation, unbound LPS was removed, and the wells were then washed three times with PBS. The wells were cut from the microtiter plates, and bound ^{125}I-LPS determined in a gamma counter (LKB Automatic Gamma Counter, LKB Instruments, Inc., Gaithersburg, MD). Assays were performed in triplicate wells.

Binding of an LPS Photoaffinity Probe to Hb—^{125}I-LPS-ASD photoaffinity probe (0.1 μCi) (prepared as described above) containing 2 μg of LPS in PBS was incubated in the dark with αα Hb (10 μg in PBS) for 30 min at 37 °C. Control incubations contained excess nonradiolabeled *S. minnesota* 595 LPS (200 μg) as a blocking agent to demonstrate inhibition of specific binding. Cross-linking was accomplished by photolysis with shortwave UV irradiation (254 nm) (UVGC-25 lamp; UVP Inc., San Gabriel, CA) at a distance of 1 cm for 15 min. Samples were reduced with 2-mercaptoethanol, electrophoresed in acrylamide in the presence of SDS, and subjected to autoradiography, as described previously (23). Hemoglobin-associated ^{125}I was determined by excising Coomassie Blue-stained protein bands and counting the associated counts/min in a gamma counter.

Ultrafiltration—Ultrafiltrations were performed using XM 100 (100-kDa cut-off) ultrafilters (Amicon Division, W.R. Grace, Danvers, MA) and ultrafree-PFL polysulfone 300 (300-kDa cut-off) ultrafilters (Millipore Corp., Bedford, MA). Filters with holders were washed with pyrogen-free 0.9% NaCl until filtrates had less than 1 ng/ml LPS as determined by the LAL test (see above). 0.9 ml αα Hb, αα HbCO, or HbA_0 (diluted to 96, 96, and 84 μg/ml, respectively, with pyrogen-free 0.9% NaCl) was incubated with 0.1 ml *E. coli* O26:B6 (W) or *P. mirabilis* S1959 LPS (each 50 μg/ml in 0.9% NaCl; 5 μg/ml, final concentration) for 30 min at 37 °C. Mixtures then were filtered manually with a 3-ml syringe (according to the directions of the filter manufacturers) at room temperature, using the 300- or 100-kDa cut-off filters. LPS concentrations in filtered solutions of Hb, Hb and LPS mixtures, or LPS alone were determined by the chromogenic LAL test (described above), using starting mixtures of Hb/LPS or LPS alone for the standard curve. Utilization of the starting mixtures for the standard curves corrected for any potential change in LPS biological activity that could occur in the presence of Hb. Hb protein concentrations were determined by the BCA protein assay (Pierce Chemical Co.). The mean values of three filtration experiments are presented.

Sucrose Centrifugation of LPS and Hb—Sucrose (4 or 20% in pyrogen-free water) was rendered endotoxin-free by filtration through an immersible CX-10 (10 kDa cut-off) ultrafiltration membrane (Millipore Corp., Bedford, MA), and 12-ml continuous sucrose gradients (4–20%) were prepared. ^{14}C-Labeled *S. typhimurium* LPS (0.005 μCi) was added to αα Hb, and the mixtures were incubated for 30 min at 20 °C. 0.1 ml of the mixture (which contained 0.002 μCi) was layered above the sucrose and centrifuged at 52,000 × *g* for 4 h in a Sorvall RC70 centrifuge and T641 swinging bucket rotor (DuPont). Following centrifugation, the tubes were then punctured and 0.4-ml fractions were collected. Hb was detected by absorbance at 405 nm. LPS was quantified by scintillation counting after samples were diluted 10-fold in fluor (Formula A-989, DuPont NEN), in a Tracor Analytic Liquid Scintillation System (Tracor Analytic, Elk Grove Village, IL). For samples containing hemoglobin, quenching of ^{14}C-LPS was reversed as follows: 0.1 ml aliquots of fractions were diluted 10-fold in water (to 1 ml, final volume), and 1 ml of Solvable (DuPont NEN) was added. These mixtures were incubated at 60 °C for 1 h, and then 0.3 ml 25% H_2O_2 was added. After 30 min of additional incubation at room temperature, samples were pale yellow in color and could be analyzed for radioactivity. Recovery of spiked radioisotope in preliminary experiments to determine the effectiveness of the decolorizing procedure demonstrated >98% detection of previously added radioactivity.

Unbound LPS also was separated from Hb·LPS complexes (and free Hb) by centrifugation through a fixed concentration of sucrose. αα Hb, αα HbCO, HbA_0, and HSA (each diluted to 10 mg/ml) were each added to ^{14}C-labeled *S. typhimurium* LPS (0.005 μCi), and the mixtures were incubated for 30 min at 20 °C. 0.3-ml aliquots of LPS/protein mixtures, LPS alone, or protein alone then were layered over 2 ml of 5% pyrogen-free sucrose and centrifuged at 2,900 × *g* for 30 min at 20 °C in a Sorvall RC-5 centrifuge (DuPont). Unbound LPS predominantly sedimented to the bottom of the tube under these conditions, whereas proteins (both in the presence and absence of LPS) remained above the sucrose layer. Following centrifugation, the solutions were separated into top (0.7–0.9 ml, including the 0.3-ml sample volume plus approximately 0.5 ml at the sample/sucrose interface), middle (0.3–0.6 ml), and bottom (0.5–0.8 ml) fractions. Hb was detected by absorbance at 405 nm, and LPS was detected by scintillation counting, as described above.

Nondenaturing Polyacrylamide Gel Electrophoresis of Hb/LPS Mixtures—Samples of ^{14}C-LPS (9,000–15,000 cpm total), αα Hb (50 μg), or αα Hb/LPS mixtures were electrophoresed in the absence of SDS in 12% polyacrylamide gels (24) for 1 h at 200 V. Following electrophoresis, the unstained gel was dried and cut into 3-mm pieces, and then each gel piece was analyzed for Hb by absorbance at 405 nm and for LPS by scintillation counting, as described above.

Ethanol Precipitation of Hb and Hb/LPS Mixtures—Insolubility of LPS in ethanol was utilized to obtain Hb complexed to LPS. 25 μl of αα Hb (2.4 μg), αα HbCO (2.4 μg), or HbA_0 (2.1 μg) and 25 μl of *E. coli* O26:B6 (W) or *P. mirabilis* S1959 LPS (25 μg each) were incubated in microtiter plate wells for 30 min at 37, 20, or 4 °C. 100 μl of ethanol (100%) then was added to each well (final concentration, 67%), and the incubations were continued for an additional 30 min at their respective temperatures of incubation (*i.e.* 37, 20, or 4 °C). The mixtures then were centrifuged at 800 × *g* for 30 min in a Sorvall GLC-2 centrifuge (DuPont), supernatants were removed, and the precipitates were resuspended in 50 μl of 0.9% NaCl. The concentrations of Hb in the resuspended sediments were determined by protein assay, and LPS concentrations in the resuspended precipitates were determined by the phenol-concentrated H_2SO_4 method (25), with galactose as the standard.

RESULTS

Binding of LPS to Hb-coated Microtiter Plate Wells—LPS bound to Hb-coated wells in a saturable manner (Fig. 1). Bind-

Chapter 12

Hemoglobin-Endotoxin Interactions

Jack Levin, M.D., Robert I. Roth, M.D., Ph.D., Wieslaw Kaca, Ph.D., Minoru Yoshida, M.D. and Donghui Su, M.D.

12.1 Introduction

Toxicities of hemoglobin (Hb) solutions, which have been demonstrated in numerous animal resuscitation models, prominently include fever, hypertension, thrombocytopenia, activation of the complement and coagulation cascades, disseminated intravascular coagulation with parenchymal organ damage, reduced tolerance to sepsis, susceptibility to bacterial infections, reticuloendothelial cell blockade and lethal toxicity (Bolin *et al.* 1983, Bornside, Bouis, and Cohn 1970, Brandt, Frank, and Lichtman 1951, Feola *et al.* 1988a and 1988b, Feola *et al.* 1990, Marks *et al.* 1989, Savitsky *et al.* 1978, Smith *et al.* 1990, White *et al.* 1986a). In addition, recent clinical trials of cross-linked Hb have been associated with production of hypertension and gastrointestinal dysmotility. Of particularly great current interest is the recent demonstration that injection of non-lethal doses of gram-negative bacteria into animals produced 50% and 100% mortality when the animals had been pre-infused with either native or cross-linked preparations of cell-free Hb, respectively (Griffiths *et al.* 1995). *In vitro*, Hb has been shown to stimulate tissue factor production by mononuclear cells (Smith and Winslow 1992), cause endothelial cell injury (Feola *et al.* 1989) and to activate complement (Smith and Winslow 1992). These *in vivo* and *in vitro* effects are characteristic of bacterial endotoxins (lipopolysaccharide, LPS). Investigations of the possibility that LPS may contribute to the observed side effects of Hb infusions have been a major focus of our laboratory during the past several years, and a significant role for LPS in Hb toxicity has been suggested by our studies.

One of the most critical aspects of LPS toxicity is the high *in vivo* potency of LPS, even at very low concentrations (pg/ml). LPS is a potentially ubiquitous contaminant during the preparation of Hb-based resuscitation fluids, and even low levels of LPS contamination become a major

clinical concern when large volumes of resuscitation solutions are required for infusion. In addition, physiologically significant levels of LPS are present in the circulating blood in a variety of clinical conditions, including sepsis, hepatic injury, hypotension, and damage to the gastrointestinal tract. Because many clinical circumstances for which Hb-based resuscitation fluids would be administered are likely to be associated with shock and hypoxia (pathological states that lead to deterioration of mucosal barriers and hepatic function), significant concentrations of endotoxin would be expected to be present in the circulation of many patients. Since there is increasing evidence that cell-free Hb and LPS synergistically produce toxicities, the infusion of Hb-based resuscitation fluids may potentiate the toxicity of pre-existing endotoxemia (or of endotoxemia that subsequently occurs when Hb remains present in the plasma), thus compounding the problem of the high intrinsic biological potency of LPS. *In vivo*, synergistic activation of coagulation and production of lethality result from the co-infusion of LPS and Hb compared to the toxicity of LPS or cell-free Hb alone (White *et al.* 1986b). We have shown that LPS clearance *in vivo* is retarded in the presence of hemoglobinemia. LPS biological effects in vitro, such as activation of coagulation mechanisms (both the direct activation of coagulation cascades and the production of monocyte and endothelial cell-derived procoagulant activity), can be enhanced up to one-hundred fold by cell-free Hb. Furthermore, rates of Hb oxidation to methemoglobin and hemichromes are dramatically increased in the presence of LPS. Thus, the ability of cell-free Hb to bind LPS produces complexes that result both in enhancement of the biological activities of LPS and degradation of Hb.

Our experience in the field of blood substitute research has been with Hb solutions, including both native human HbA_0 and cross-linked Hb (human Hb, $\alpha\alpha$ cross-linked using bis(dibromosalicyl) fumarate (DBBF)). Investigations in our laboratory during the past several years have led to an understanding of the complex contributions of LPS to the observed toxicities of Hb solutions. Initial experiments suggested the possibility that Hb was a previously unrecognized LPS binding protein. Subsequently, detailed experiments documented the formation of Hb-LPS complexes, characterized the complexes, and identified consequences of the LPS-Hb interaction that might contribute to toxicity.

12.2 Demonstration That Hemoglobin is an LPS Binding Protein

An extensive series of experimental approaches have been utilized to document that mixtures of LPS and Hb produce stable complexes (Kaca, Roth and Levin 1994). In all experiments, equivalent results were obtained using either purified native, unmodified human HbA_0 or cross-linked Hb prepared as a potential red cell substitute. Direct evidence of

saturable binding of LPS to immobilized Hb was obtained (Figure 12.1). The calculated K_d (4.7×10^{-4} g/liter [3.1×10^{-8} M, assuming a monomer molecular mass of 1.5×10^4 for *E. coli* LPS] based on the microtiter plate

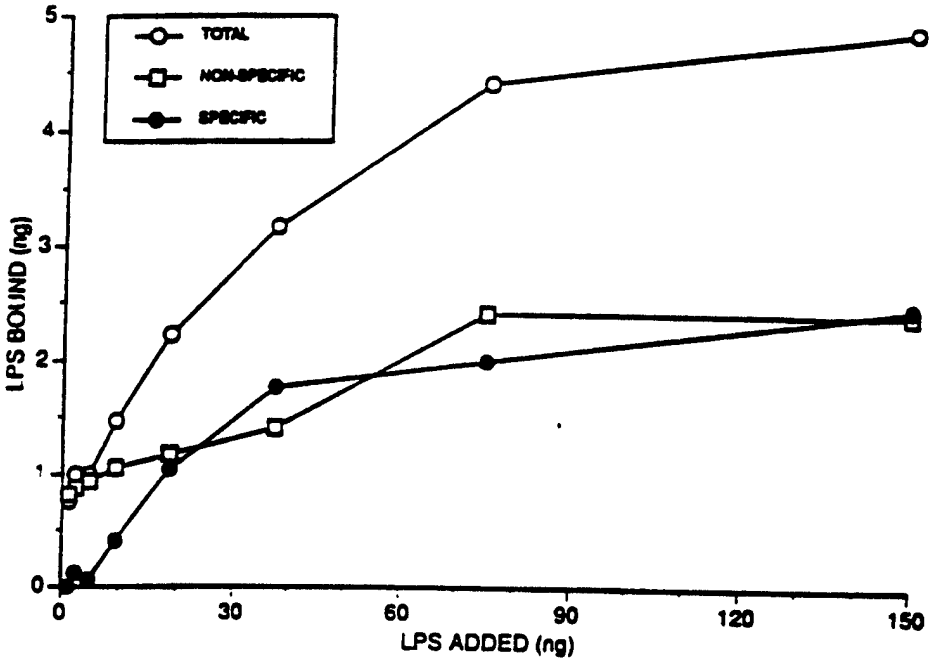

Figure 12.1. Binding of LPS to immobilized Hb. ααHb (1 μg/well) was immobilized in microtiter plate wells, and ^{125}I-LPS was added. Bound LPS was determined by gamma counting, and specific binding was calculated by subtracting bound ^{125}I-LPS in wells without Hb.

binding assay and 6.3×10^{-4} g/liter based on a sucrose centrifugation assay) indicated that the interaction between Hb and LPS is of moderate affinity. Complex formation also was demonstrated by affinity-labeling of Hb with a photoactivatable form of LPS (Figure 12.2). Using density gradient centrifugation, co-migration of LPS with Hb was shown, and it was demonstrated that the sedimentation velocity of LPS was decreased in the presence of Hb preparations (Figure 12.3). This indicated that there had been disaggregation of LPS and formation of lower density Hb-LPS complexes. Additional evidence of LPS dissociation was obtained by non-denaturing polyacrylamide gel electrophoresis which demonstrated that LPS, when complexed with Hb, entered the gel and co-migrated with Hb, whereas LPS alone remained within the stacking gel (Figure 12.4). Ultrafiltration experiments demonstrated that LPS, which alone in aqueous solutions has a very high molecular weight (typically $\geq 10^6$ daltons), co-filtered with Hb through 300 kDa and 100 kDa membranes (Table 12.1). Whereas only 10-15% of LPS alone was filterable through the 300 kDa membrane and LPS alone was not filterable at all through the 100 kDa membrane. In the presence of Hb, 87-97% of LPS was filtered through the 300 kDa membrane and 64-72% through the 100 kDa membrane. These data provide further evidence that Hb greatly decreased the aggregate molecular weight of LPS.

Table 12.1 Ultrafiltration of E. coli O26:B6 and P. mirabilis S1959 LPS, Hb, and LPS-Hb mixtures*.

filter	E. coli LPS filtered (%) 300 kDa** filter	P. mirabilis LPS filtered (%) 100 kDa filter	P. mirabilis LPS filtered (%) 300 kDa**
LPS alone	10.2 ± 2.3	0	15.6 ± 5.6
ααHb alone	0***	0	0
ααHb + LPS	87.3 ± 8.0	63.6 ± 18.7	97.1 ± 1.5
ααHbCO alone	0	0	0
ααHbCO + LPS	89.3 ± 1.5	71.1 ± 4.0	90.9 ± 4.5
HbA_0 alone	0	0	0
HbA_0 + LPS	88.1 ± 3.7	71.6 ± 8.8	93.5 ± 8.6

* Each experiment was performed three times and the mean ± 1 SD is shown. Percent of LPS filtered was determined with the chromogenic LAL test. LPS was quantified with reference to standard curves consisting of the respective LPS/protein mixture prior to filtration.
** Molecular weight cut-off of the filter.
*** Lack of detectable LPS indicates that the starting preparations of Hb were endotoxin-free.

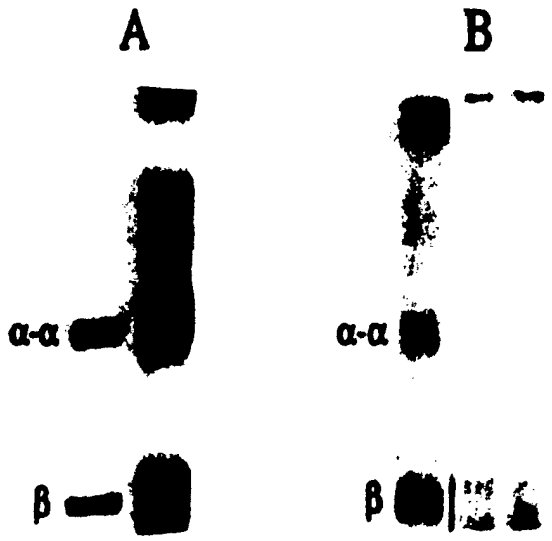

Figure 12.2. Photoaffinity labeling of Hb with ^{125}I-LPS-ASD. ^{125}I-LPS-ASD was incubated with ααHb, photolyzed with UV light, and electrophoresed in SDS and 2-mercaptoethanol. Following electrophoresis, the gel was stained with Coomassie blue (A, left lane), dried, and subjected to autoradiography (A, right lane). Another photoaffinity-labeled ααHb preparation from a separate experiment is shown (B, left lane), along with controls that consisted of an incubation mixture containing 100-fold excess unlabeled LPS as a blocking agent to demonstrate inhibition of specific binding (B, middle lane) and ^{125}I-LPS-ASD alone (B, right lane).

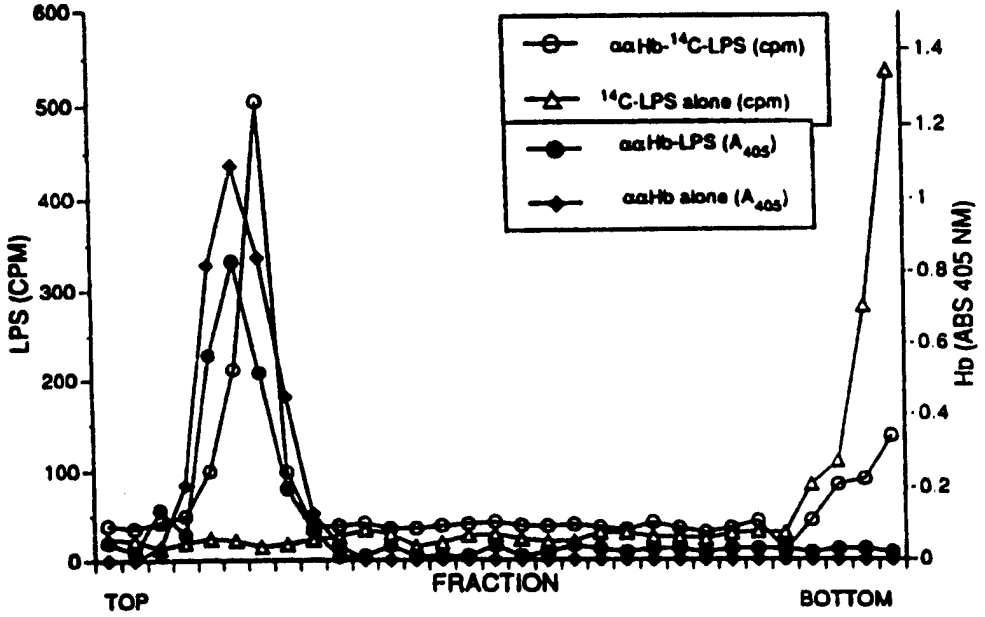

Figure 12.3. Sucrose density centrifugation of LPS-Hb. ^{14}C-LPS was incubated with ααHb (100 mg/ml), and the mixture centrifuged through a 4-20% continuous sucrose gradient. 0.4 ml fractions were assayed for hemoglobin by absorbance at 405 nm (closed symbols), and for LPS by scintillation counting (open symbols).

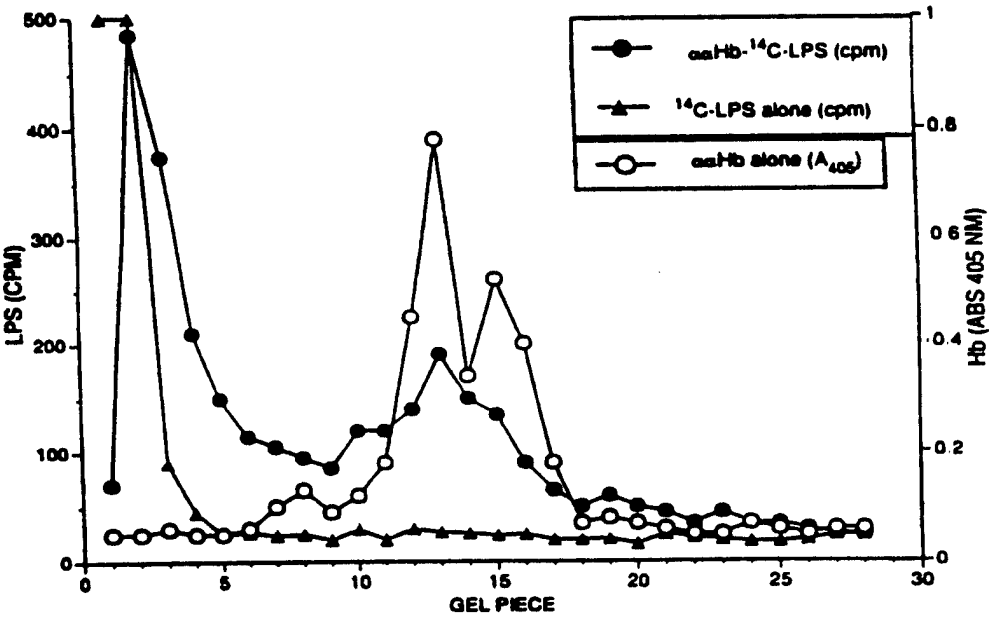

Figure 12.4. Electrophoresis of LPS and Hb. ^{14}C-LPS was incubated with ααHb, and the ααHb-LPS mixture or LPS alone was electrophoresed in polyacrylamide in the absence of SDS, as described in the text. ^{14}C-LPS was measured by scintillation counting of gel pieces (closed symbols), and ααHb was monitored by absorbance at 405 nm (open circles).

Conversely, Hb-LPS complex formation has been shown to result in Hb denaturation, with production of methemoglobin and hemichromes (Figure 12.5) (Kaca *et al.* 1995). Degradation of Hb by LPS is time (Figure 12.6) and LPS-concentration dependent. There are structural changes indicative of Hb oxidation as demonstrated by circular dichroic analysis between 210-600 nm (Figure 12.7). However, there is no demonstrable change in the overall tertiary structure of the globin molecule.

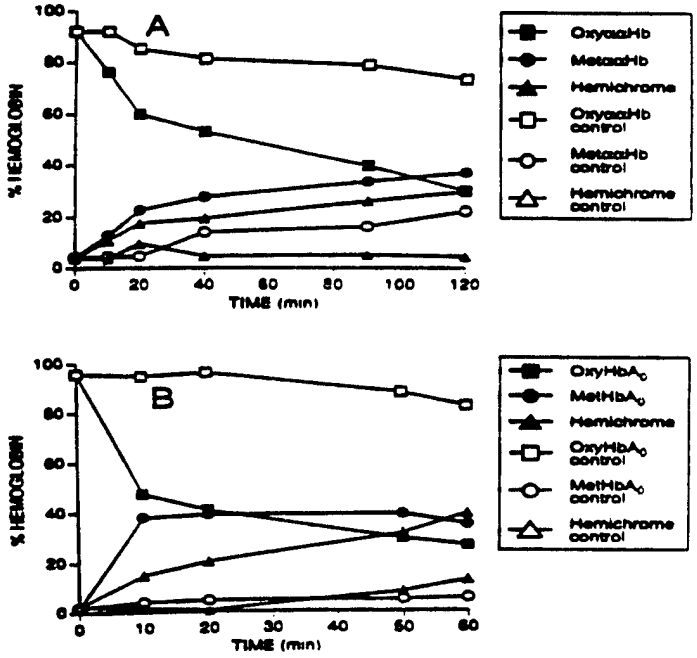

Figure 12.5. Time-dependent conversion of ααHb (A) and HbA_0 (B) to metHb and hemichromes in the presence of *S. minnesota* 595 OH37 LPS (0.3 mg/ml and 0.8 mg/ml LPS incubated with ααHb and HbA_0, respectively). Percentages of oxyHb, metHb, and hemichromes were determined according to the method of Winterbourn (1990). Open symbols, Hb alone; closed symbols, Hb + LPS.

12.2.1 P_{50} Measurements

The oxygen affinity of Hb was measured in the absence and presence of LPS in order to evaluate the possible influence of LPS binding on Hb function (Table 12.2) (Kaca *et al.* 1995). These measurements were made after a 2 hr incubation period, a time sufficient to result in Hb-LPS complex formation (Kaca *et al.* 1994), but prior to the formation of substantial quantities of oxidized Hb species unable to bind oxygen. One mg/ml Hb (16 μM) and 1 mg/ml of each LPS were utilized because the two components of Hb-LPS complexes are of approximately equal concentration by weight and little unbound Hb is calculated to be present. P_{50} values for ααHb (26.6 mm Hg) and HbA_0 (9.6 mm Hg) were slightly decreased by both smooth and rough LPSs (Table 12.2). Non-cross-linked cell-free HbA_0, which exhibited high oxygen affinity (P_{50} = 9.6 mm Hg) similar to that measured with lysed whole blood (P_{50} = 10.0 mm Hg; data not shown), best demonstrated the small trend toward higher oxy-

gen affinity when in the presence of LPS (P_{50} = 7.3 mm Hg in the presence of OH37 LPS). Thus, there is little change in oxygen affinity of Hb when complexed to LPS.

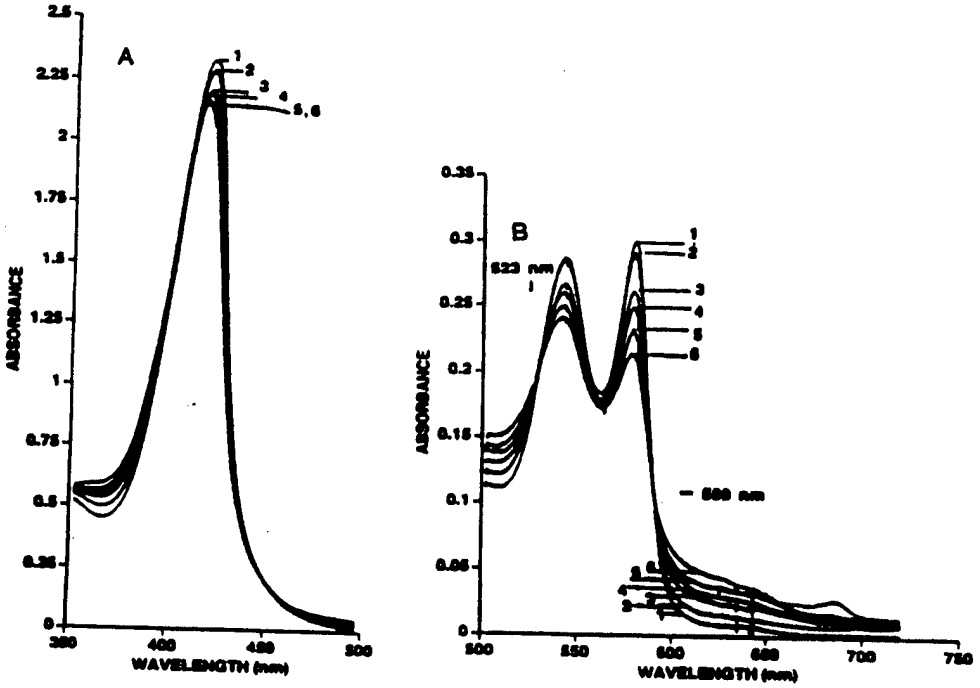

Figure 12.6. Time course of changes in the hemoglobin absorption spectrum in the presence of LPS. ααHb (21 μM in PBS, pH 7.4) was incubated at 37°C in the presence of 0.3 mg/ml *S. minnesota* 595 OH37 LPS, and absorbance spectra in the Soret (A) and visible (B) regions of the Hb spectrum were obtained at various times of incubation. 1 - initial spectrum of ααHb alone; 2 - 10 min; 3 - 20 min; 4 - 40 min; 5 - 90 min; 6 - 120 min. The sample cuvette contained Hb in PBS with or without LPS, and the reference cuvette contained PBS (for ααHb spectra alone) or LPS alone (0.3 mg/ml in PBS) (for ααHb-LPS mixture spectra). The arrows indicate the apparent isosbestic points.

Table 12.2 p50 values for Hb and Hb/LPS complexes*.

	P50
ααHb alone	26.6
ααHb + LPS[a]	25.1
ααHb + LPS[b]	25.6
HbA_0 alone	9.6
HbA_0 + LPS[c]	8.7
HbA_0 + LPS[d]	7.3

*Oxygen affinity measurements were obtained for cross-linked (ααHb) and native (HbA_0) hemoglobins alone or in the presence of LPS after a 2 hour incubation at 37°C. Measurements were obtained prior to the production of oxidized Hb species P50 was determined utilizing both smooth and rough LPSs: [a]*P. mirabilis* 03 (smooth) LPS; [b]*S. minnesota* Re 595 (rough) LPS; [c]*E. coli* 026 (smooth) LPS; [d]*S. minnesota* 595 OH37 (rough) LPS. Equal concentrations of Hb and LPS were utilized (each 1 mg/ml prior to dilution in Hemox buffer.

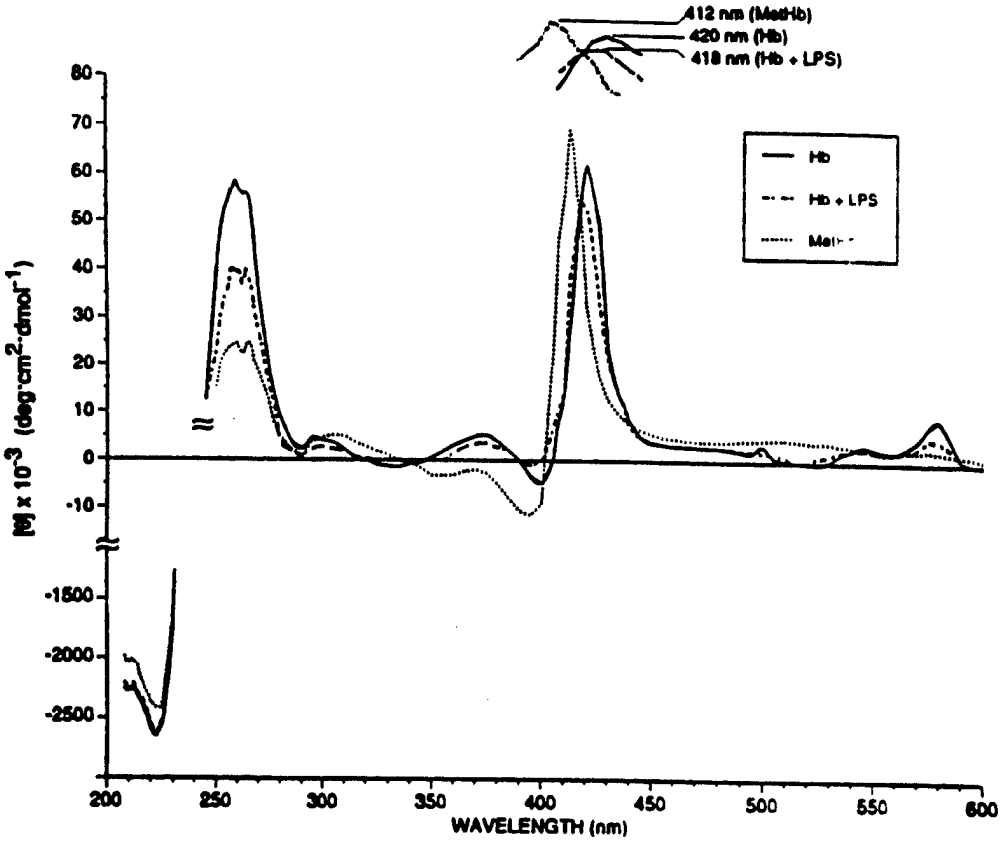

Figure 12.7. Circular dichroic (CD) spectra of Hb in the absence and presence of LPS. CD spectra were measured at room temperature between 200 nm and 600 nm for ααHb alone (13.8 μM heme, ambient oxygenation), ααHb/LPS (13.8 μM heme, 0.5 mg/ml *S. minnesota* 595 OH37 LPS, after 2 hr incubation at 37°C), and metααHb (31.5 μM heme). Measurements in the far UV region were made with samples diluted 5 to 10-fold. A 1 cm path length cell was utilized for measurements between 250-400 nm and 430-600 nm, and a 0.2 cm path length cell was utilized for measurements of the major Soret (400-440 nm) and far UV (210-250 nm) regions. Ellipticities, [q], are expressed on a molar heme basis. Wavelengths for the Soret peak maxima are identified on inset tracings presented with an expanded *x*-axis.

12.3 Effect of Hb on LPS Clearance *in vivo*

LPS clearance in rabbits was shown to be delayed in the presence of Hb (free Hb levels were 2 g/dL, which produced a 15% increase in total circulating Hb) compared to LPS clearance in animals given equivalent doses of human serum albumin (HSA) or NaCl (Figure 12.8). (Yoshida, Roth and Levin 1995). The intravascular retention of injected ^{125}I-LPS during the 30 min period analyzed was significantly longer in the LPS + Hb group than in the LPS + NaCl or LPS + HSA groups, especially during the initial 10 min. The intravascular half-life ($T_{1/2}$) of LPS in the LPS + NaCl control, LPS + HSA control, and LPS + Hb groups was 2.8, 4.0, and 4.9 min; the area under the curve was 1,369 ± 483, 1,594 ± 360,

and 1,731 ± 481 (ng/ml x min, mean ± SD); and the total body clearance was 24.7 ± 9.2, 20.1 ± 5.4, and 18.9 ± 6.0 (ml/min, mean ± SD), respectively. The proportion of LPS associated with blood cells was very small at the initial 1 min time period, and decreased even further during the 30 min period analyzed. Over 96% of injected LPS was associated with the cell-free plasma, with 51-54% of LPS in the apoprotein fraction at the initial time point, and 35-37% in the high density lipoprotein (HDL) fraction. The proportion of LPS increased significantly in the HDL fraction and decreased significantly in apoproteins during the 30 min period analyzed. However, there were no differences between the three groups (Yoshida, Roth and Levin 1995, Roth, Levin and Levin, 1993). The liver was the main distribution site (74%) of injected LPS, among the six organs evaluated (liver, kidney, lung, spleen, adrenal, and heart). In the Hb group, the accumulation of ^{125}I-LPS in the spleen was significantly lower than in the HSA group. The synergism of the in vivo toxicity reported for LPS and Hb may be due, in part, to the decreased rate of intravascular clearance of endotoxin.

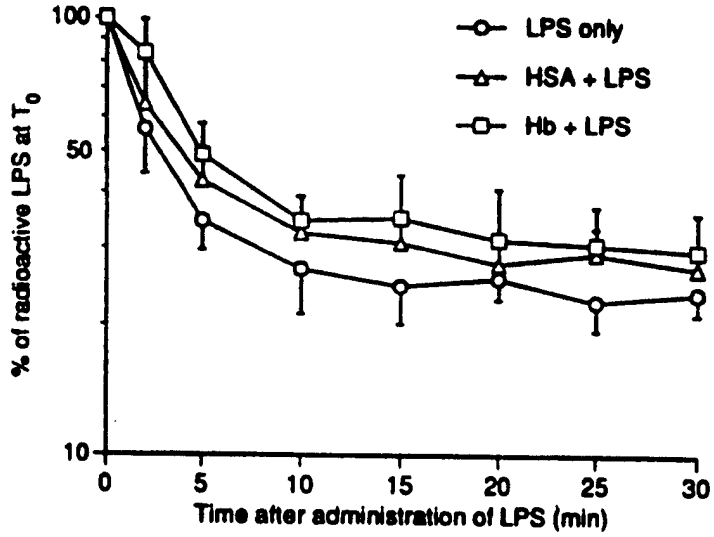

Figure 12.8. Intravascular clearance of ^{125}I-LPS after intravenous injection into rabbits: LPS only (E), LPS administered immediately following a 10 minute infusion of HSA (C), or following hemoglobin (Hb) (G). The numbers of animals in each group were 6, 6, and 5, respectively. Values represent the mean percent of the level of radioactive LPS in whole blood at T_0 ± SD.

12.4 Demonstration That Hemoglobin Enhances the Biological Activity of LPS

12.4.1 Limulus amebocyte lysate activation

The effect of Hb on the biological activity of LPS was initially investigated using Limulus amebocyte lysate (LAL), the most sensitive *in vitro*

assay for LPS. LAL, a preparation from the blood cells of the horseshoe crab *Limulus polyphemus,* contains an LPS-activated coagulation cascade that is sensitive to pg/ml concentrations of LPS. Each of a variety of LPS preparations spiked into solutions of LPS-free Hb demonstrated greatly increased activation of LAL in comparison to identical concentrations of LPS assayed in saline (Figure 12.9) (Kaca *et al.* 1994). This result is of great interest because LAL activation is an excellent model for the intravascular coagulation, which is commonly seen in humans during endotoxemia, and which has been described repeatedly during infusion of hemoglobin solutions in animals. Further experiments demonstrated that LAL enhancement activity was dependent on the concentration of Hb; LPS biological activity was enhanced >1000-fold at the concentrations of Hb that would be achieved *in vivo* for purposes of resuscitation. Pertinently, similar Hb concentrations have been detected in plasma following hemolysis associated with endotoxemia.

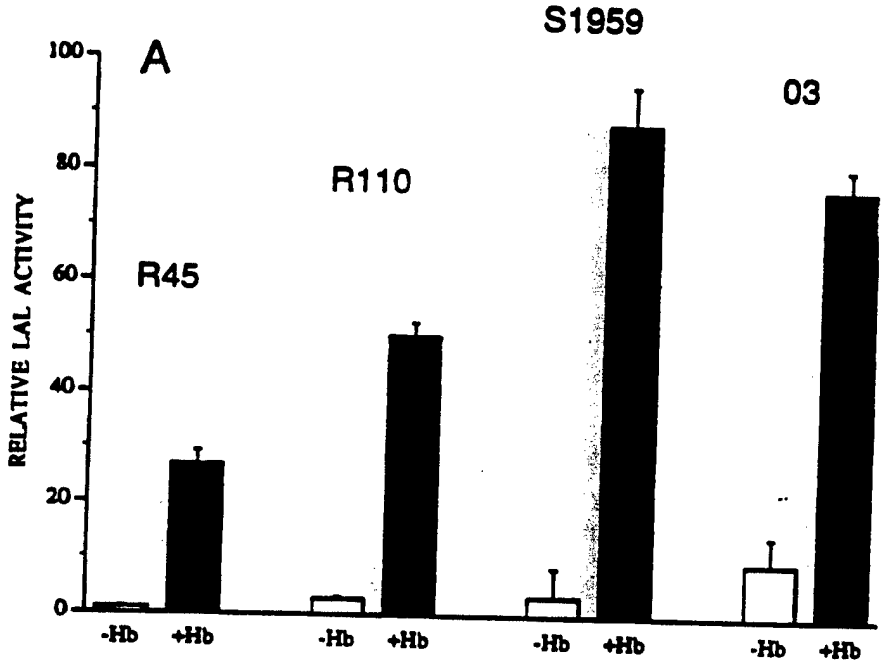

Figure 12.9. Enhancement by hemoglobin of the activation of LAL by *Proteus* LPS. LAL reactivities of LPS (500 ng/ml) in the absence (-Hb) or presence (+Hb) of αα–hemoglobin (1 mg/ml) were determined with the chromogenic LAL assay. To determine relative LAL activities, a standard curve of *P. mirabilis* R45 LPS was prepared, which related absorbance to LPS concentration. Using this standard curve, the absorbance for each sample (LPS alone or LPS-Hb) was converted into the equivalent R45 LPS concentration. 500 ng/ml R45 LPS was assigned a relative LAL activity of 1. Samples were assayed with eight replicates, and results are expressed as the mean ± 1 S.D.

Many of the LPS preparations studied had poor aqueous solubility and were visually turbid (especially *S. minnesota* 595 LPS, lipid A and monophosphoryl lipid A (MPL), and *P. mirabilis* (R110). Hb enhancement of

LPS biological activity was a prominent feature of some of these LPS and partial structures, suggesting that a possible mechanism for the Hb enhancement effect was due to increased LPS solubility. Therefore, we compared turbidity and the LAL biological activity of these LPS in the absence and presence of Hb (Kaca *et al.* 1994). With increasing concentrations of ααHb, *P. mirabilis* R110 and *S. minnesota* 595 LPS each demonstrated a concomitant progressive decrease in turbidity and increase in LAL biological activity (Figure 12.10).

In order to further establish the generalized nature of the Hb enhancement effect, we studied the effect of ααHb on biological activities of several other LPSs, including LPSs from different bacterial species. Prominent, and identical, extents of enhancement by both ααHb and ααHbCO in the LAL assay were shown with three defined salts of *E. coli* 026:B6 (smooth LPS), *i.e.*, the calcium, sodium and triethylamine forms, suggesting that the specific cations bound to LPS did not influence the Hb enhancement process. Enhancement of LPS biological activity also was demonstrated with a smooth Salmonella LPS (*S. abortus equi*) and a rough *E. coli* LPS (Re F515), but was not observed with non-toxic *Rhodobacter spheroides*, *Rhodobacter capsulatus* and *Rhodopseudomonas viridis* LPSs.

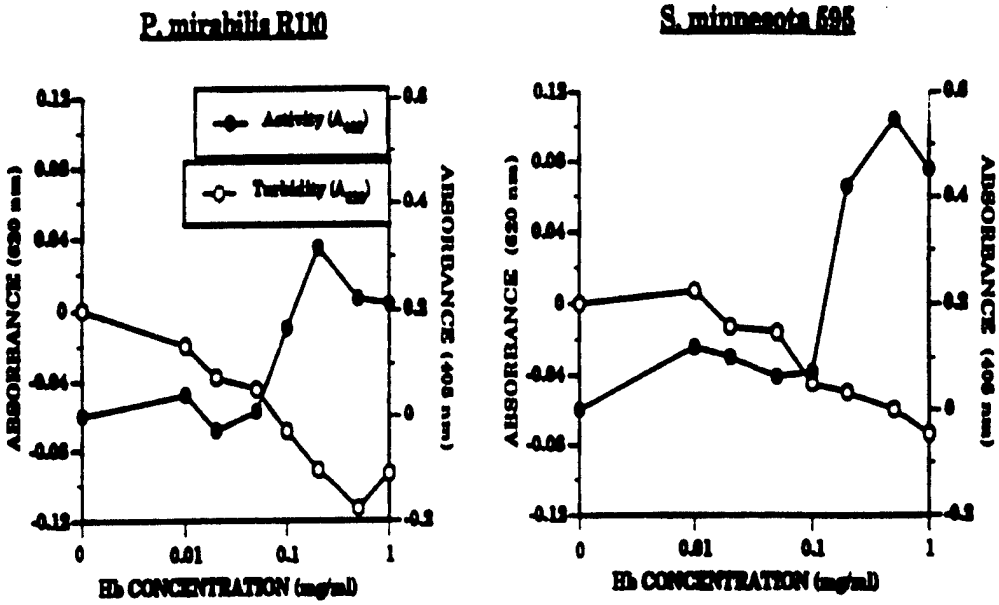

Figure 12.10. Turbidity and biologic activities of LPS in the absence and presence of Hb. Various concentrations of αα–Hb (from 0.01 to 1.0 mg/ml) were added to LPS (final concentration, 1 mg/ml) in microtiter plate wells and absorbances were measured at 620 nm. The turbidity of each LPS (absorbance at 620 nm) in the absence of Hb has been designated as 0, and the change in absorbance induced by Hb is shown. Absorbances due to Hb have been subtracted. Actual baseline LPS absorbances were as follows: *P. mirabilis* R110, 0.21; *S. minnesota* R 595, 0.12. LAL then was added to each well and chromogenic activities determined at 405 nm.

12.4.2 Tissue factor production

To further investigate the ability of Hb to modify LPS-activated coagulation, we evaluated the effect of Hb on LPS stimulation of mononuclear cell procoagulant activity (*i.e.*, tissue factor, TF). This is another coagulation-based assay for LPS activity which is quantitative (as is the LAL assay), and which is known to correlate well with LPS activity as determined by LAL. A Hb concentration-dependent enhancement of LPS-stimulated procoagulant activity in mononuclear cells was observed (Figure 12.11) (Roth *et al.* 1993).

Since Hb has the ability to increase the production of TF by mononuclear cells, we reasoned that vascular endothelium might demonstrate a similar response. Cultured human umbilical vein endothelial cell (EC) monolayers were incubated with LPS, in the presence and absence of Hb, and the generation of EC procoagulant activity (TF) was determined. LPS alone (0.001 mg/ml to 10 mg/ml) caused a concentration dependent increase in production of EC TF activity, compared to the TF produced by unstimulated cells. Hb resulted in augmented production of TF in response to LPS (Figure 12.12). (Roth 1994). This enhancement was demonstrated with both native and cross-linked Hbs, and was shown to be concentration dependent between 0.1 mg/ml and 100 mg/ml Hb. The production of TF activity was completely inhibited by Actinomycin D or cycloheximide, indicating a requirement for new protein synthesis.

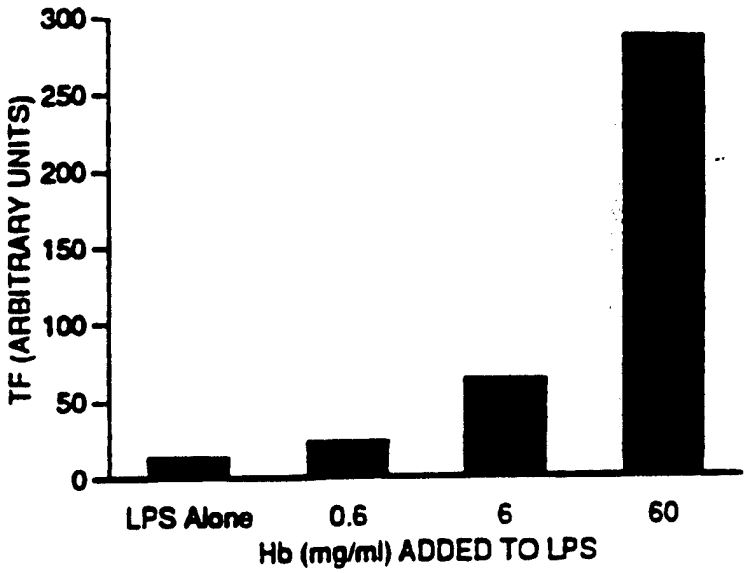

Figure 12.11. Tissue factor (TF) production by human mononuclear cells. Human mixed mononuclear cells were incubated with LPS in the presence of various concentrations of endotoxin-free Hb (0.6-60 mg/ml). TF generated by LPS alone and the Hb-LPS mixtures was determined with a plasma recalcification assay. The contribution of the Hb alone(at each concentration, respectively) to the total TF generated by the mononuclear cells was subtracted from the measured total.

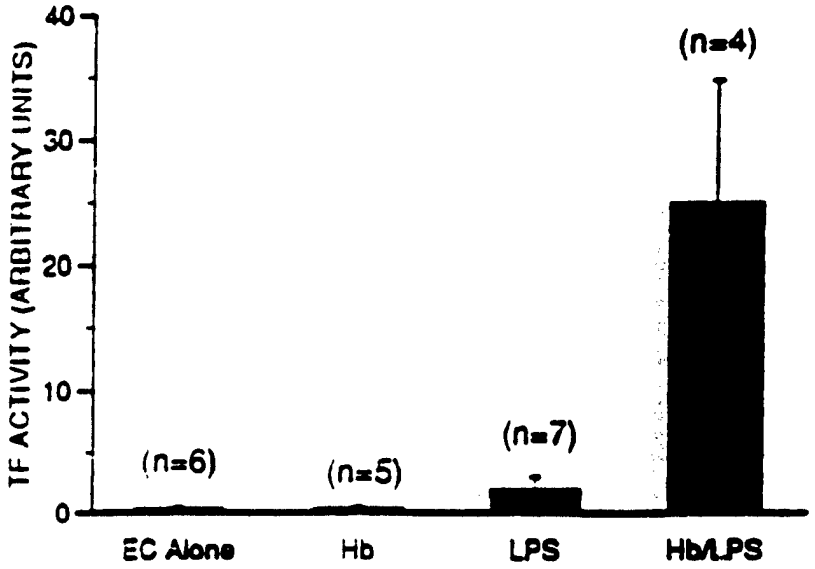

Figure 12.12. Effect of Hb on the production of endothelial cell tissue factor (TF) in response to LPS. Cultured human endothelial cells were incubated with Hb alone, LPS alone, or LPS in the presence of Hb. TF activities then were determined with a plasma recalcification assay.

Elevated levels of TF protein in response to Hb-LPS, as assessed by an ELISA assay, also were demonstrated. Inhibition of nitric oxide synthesis, using N-monomethyl-L-arginine (L-NMMA), resulted in attenuated TF production (10-80% decrease of TF) by the EC in response to both LPS alone and Hb-LPS.

12.4.3 Platelet adherence to endothelial cells

Because of the critical role of the vascular endothelium in promoting pathological hemostatic responses to LPS *in vivo* (LPS transforms the endothelium from an anticoagulant surface to a procoagulant surface), we also examined whether Hb modified LPS-induced platelet adherence to endothelial cells (EC). Cultured human EC monolayers were incubated with LPS, in the presence and absence of HbA_0, and the binding of radiolabeled human platelets was examined. LPS alone resulted in slightly increased binding of human platelets to ECs in culture (20% increase compared to platelet binding in the absence of LPS), and Hb-LPS complexes further increased platelet binding to ECs (35% increase compared to platelet binding in the control without LPS or Hb). Incubation of the ECs with Hb alone resulted in a slight decrease in platelet binding.

12.4.4 Complement activation

Enhancement by Hb of the biological activity of LPS in the activation of a proteolytic coagulation cascade in LAL suggested that there may be an impact of Hb on the ability of LPS to activate other protease cascades. We studied whether formation of Hb-LPS complexes altered the ability of LPS to activate and fix complement (a process thought to contribute to the in vivo toxicity of Hb in animal studies). Addition of Hb had little or no effect on the intrinsic complement fixing abilities of eight smooth endotoxins, rough endotoxins, or endotoxin partial structures (Kaca and Roth, 1995). At higher concentrations (>0.2 mg/ml), Hb by itself also was capable of fixing complement, in the absence of LPS, *via* the classical pathway of complement activation.

12.4.5 Lethality in Mice

Because of the extensive *in vitro* data we obtained demonstrating the ability of Hb to enhance the biological activity of LPS, we initiated animal experiments to determine whether LPS-induced mortality was affected by the presence of hemoglobinemia. Mice were injected i.p. with an LD50 dose of *E. coli* LPS(500 μg), and 8 hr later received an i.v. infusion of Hb (60 mg) sufficient to raise the blood Hb level by 2-3 g/dL. LPS-induced mortality at several time points was increased by Hb (Figure 12.13). Mortality in the Hb-treated mice was also noted many hours earlier than in mice which had received only LPS. Furthermore, Hb increased endotoxin-related mortality in mice whether it was infused intravenously prior to, coincident with, or subsequent to intraperitoneal endotoxin injection. Enhanced mortality was observed over a range of doses of injected LPS. At a given endotoxin dose, enhancement of mortality was dependent on the dose of Hb administered. In the presence of endotoxemia, doses of Hb ≥ 22 mg resulted in increased mortality; a dose of ≤ 11 mg did not increase mortality. Hb itself caused no mortality, and mice which received Hb alone appeared completely normal throughout the study. Other experiments suggested that an increased cytokine response and depressed reticuloendothelial cell function may have contributed to the enhanced mortality from LPS in the presence of Hb. Our observations are consistent with the previous reports that indicate the presence of free Hb in the circulation can compromise reticuloendothelial system function and increase susceptibility to bacterial infection (Eaton, Brandt and Mahoney 1982, Kay and Hook 1963a and 1963b, Kaye, Gill and Hook 1967, Litwin *et al.* 1963, Schneidkraut and Loegering 1980).

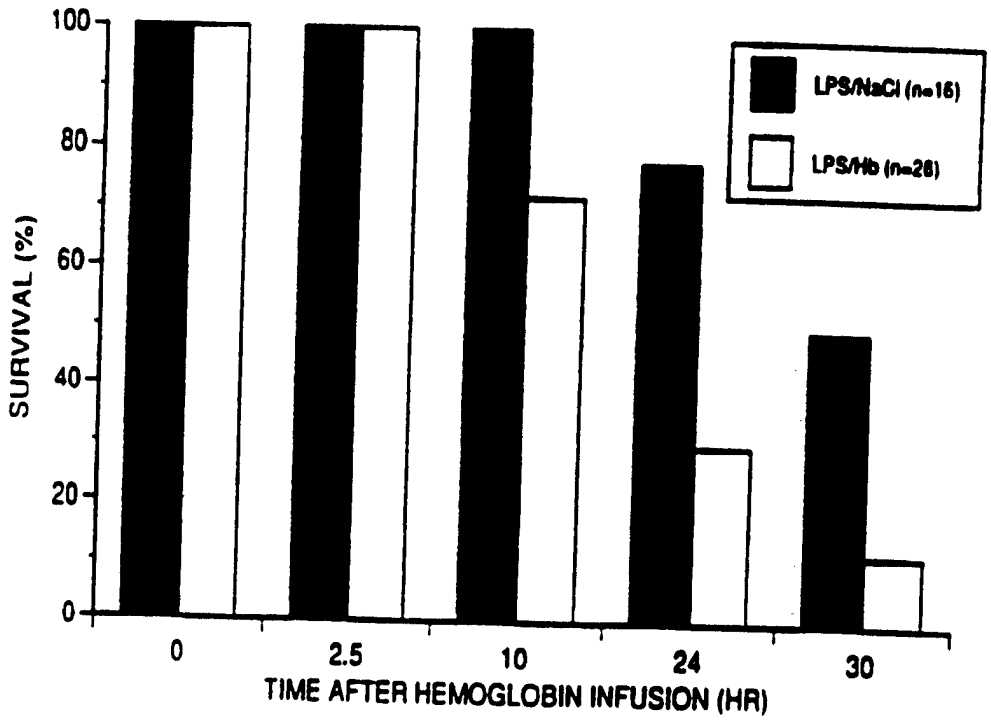

Figure 12.13. Effect of Hb on mortality from LPS. Mice were injected i.p. with an LD50 dose of LPS (500 μg/animal), followed 8 hr later by i.v. infusion of Hb (which generated a plasma Hb concentration of 2 g/dL) or 0.9% NaCl. Survival for 30 hr after Hb infusion is shown.

12.5 Conclusions

The development of non-infectious, non-antigenic stable red blood cell substitutes for human use is of great importance in both military and civilian settings. Products presently under investigation include a variety of derivatized cell-free Hb preparations, perfluorocarbon emulsions, and encapsulated Hb preparations. Safety of red cell substitutes, as well as efficacy, have been identified as critically important by the Center for Biologics Evaluation and Research (1991 and 1994). Our data suggest that hemoglobin-based blood substitutes, which are currently undergoing clinical trials (Ogden and MacDonald 1995), may intensify the potentially fatal effects of the sepsis syndrome in patients with trauma, infection or hypotension who receive hemoglobin for red blood cell replacement. Therefore, Hb should be administered to such patients with caution, and thorough serial physiological observations performed in order to detect any worsening of signs or symptoms that may be attributable to endotoxemia and the sepsis syndrome. In this regard, it will be important to measure endotoxin levels in the blood of appropriate patients, in order to detect the presence of endotoxemia during or after the administration of solutions of hemoglobin.

12.6 Acknowledgments

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Figures 12.1, 12.2, 12.3, 12.4 and Table 12.1 are from Kaca, Roth and Levin 1994.

Figures 12.5, 12.6, 12.7 and Table 12.2 are from Kaca *et al.* 1995.

Figure 12.8 is from Yoshida, Roth and Levin 1995.

Figures 12.9 and 12.10 are from Kaca *et al.* 1994.

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Blood Substitutes

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Ultrastructural changes in bacterial lipopolysaccharide induced by human hemoglobin

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Summary Recently published studies showed that human hemoglobin (Hb) forms stable complexes with bacterial endotoxin (lipopolysaccharide, LPS), and that these complexes have much greater biological activity than LPS alone. LPS in LPS-Hb complexes has greater filterability, is less turbid, and has a lower sedimentation velocity compared to LPS alone, changes consistent with smaller LPS size. To further characterize the physical changes in LPS induced by Hb, we examined the ultrastructure of LPS alone and LPS-Hb complexes by negative staining. We studied two rough (Re) LPS and one smooth LPS, before and after incubation with purified human HbA_0. *Salmonella minnesota* (Re) 595 LPS was visibly turbid, and was composed mostly of ribbon-like and mesh-like structures. In addition, this LPS contained large irregular membrane-like masses, and a few smaller discoidal particles. After incubation with Hb, none of the large mesh-like, ribbon-like or membrane-like structures remained; the resultant LPS particles were discoidal particles of 5–20 nm. *Escherichia coli* (Re) LPS alone was comprised predominantly of ribbon-like structures; after incubation with Hb, this LPS was comprised of circular membrane-like sheets (predominantly 40–200 nm). Smooth *E. coli* 055:B5 LPS alone contained heterogeneous particles of 10–100 nm. Most of the largest of these particles were disintegrated by the addition of Hb. The time course of Hb-induced LPS disaggregation was studied with *S. minnesota* LPS because the great turbidity of this LPS allowed us to monitor its disaggregation spectroscopically. Turbidity was decreased by 50% within approximately 5 h, and was not measurable by 25 h. We propose that the toxic domains of LPS (i.e. lipid A) are cryptic in native LPS aggregates and become exposed upon binding with Hb, thereby resulting in increased biological activity of LPS-Hb complexes compared to LPS alone.

INTRODUCTION

In the past several years, there has been tremendous progress in our understanding of the mechanisms of action of bacterial endotoxin (lipopolysaccharide, LPS). A large number of plasma and cell-associated LPS binding proteins have been described,[1–5] and the interactions of LPS with these proteins have been shown to be essential for maximal LPS biological activity. Recent efforts to understand the mechanisms of toxicity of infused hemoglobin (Hb) preparations that are being developed as red blood cell substitutes led to the recognition that Hb interacts with LPS with formation of stable complexes.[6,7] In several in vitro assays of LPS biological activity, Hb was shown to increase LPS potency.[7,8] Additional in vitro studies showed that LPS induces Hb oxidation.[9] In vivo, Hb also has been demonstrated to enhance LPS-induced mortality.[10] The mechanism of Hb enhancement of LPS biological activity involves LPS-Hb complex formation, as shown by direct binding assays and photoaffinity labeling of Hb with an LPS probe.[6] Complexes of Hb-LPS have

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been shown to have decreased size compared to LPS alone based on filterability through Amicon membranes and decreased sedimentation velocity.[6] These changes induced by Hb were indicative of LPS disaggregation. Characterization of these Hb-LPS complexes demonstrated that the majority were filterable through 100 kDa membranes, and the remainder were filterable through 300 kDa membranes. It would be expected that the Hb-LPS complexes of less than 100 kDa would contain no more than 3 smooth LPS monomer molecules bound per Hb (based on the 64 kDa molecular mass of tetrameric Hb and an estimated average monomer molecular mass of 12 kDa for smooth LPS). In contrast, aggregated smooth LPS alone is typically > 10^6 Da, consistent with > 100 monomer units per LPS aggregate. A rough association has been demonstrated between a decrease in LPS turbidity induced by Hb binding and an increase in LPS biological activity,[7] suggesting that LPS disaggregation may be a pathophysiologically important feature of the LPS-Hb interaction. Therefore, it was of interest to further characterize these highly active Hb-LPS complexes.

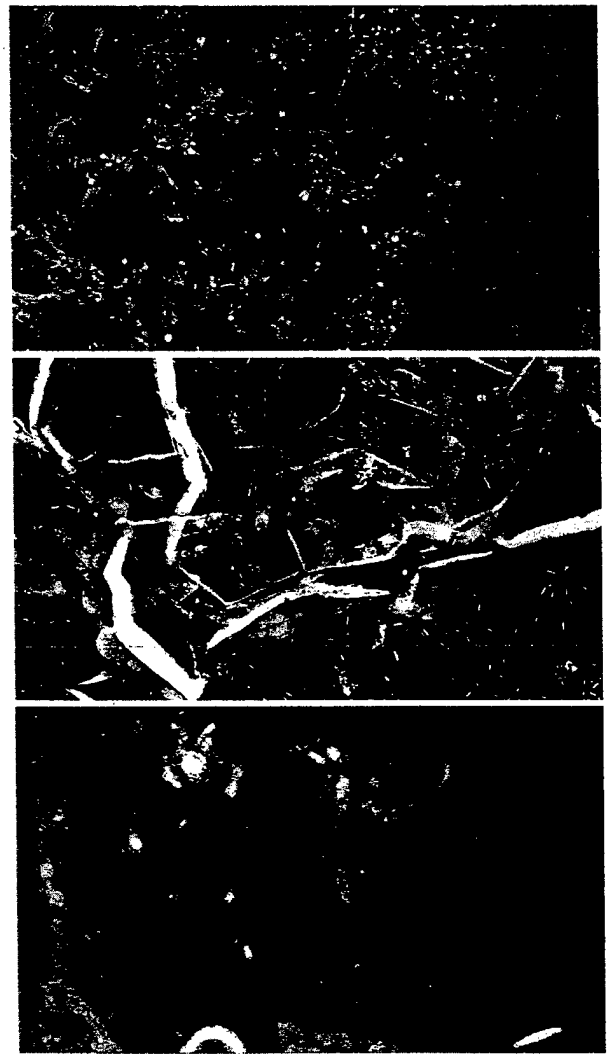

Fig. 1 **Top** Highly aggregated *S. minnesota* (Re) 595 LPS demonstrating primarily ribbon-like (arrows) and mesh-like (open arrows) structures (×60,000). **Middle** Highly aggregated Re LPS demonstrating primarily large membrane-like sheets (arrow heads) (×60,000). **Bottom** High-power image (×180,000) of ribbon-like (arrows) and mesh-like (open arrow) LPS structures.

MATERIALS AND METHODS

Reagents

Sterile, endotoxin-free water and 0.9% NaCl were purchased from Travenol Laboratories (Deerfield, IL, USA). Sterile, endotoxin-free plasticware was obtained from Beckton Dickinson (Mountain View, CA, USA). Phosphotungstic acid was obtained from Merck and Co., Inc. (Rahway, NJ, USA).

Bacterial LPS

S. minnesota (Re) LPS was purchased from List Biological Laboratories, Inc. (Campbell, CA, USA) and E. coli 055:B5 LPS from Difco Laboratories (Detroit, MI, USA). These LPS had not been electrodialyzed. E. coli (Re) LPS (TEA salt form) was the generous gift of Dr Chris Galanos, Max-Planck Institut für Immunbiologie, Freiburg, Germany.

Hemoglobin

Human HbA_0, purified by ion exchange chromatography, was provided by collaborators at the Blood Research Division of the Letterman Army Institute of Research, San Francisco, CA, USA. The Hb was 8.4 g/dl, pH 7.4 in Ringers acetate buffer, and contained < 100 pg/ml LPS as determined by LAL assay.[11]

Electron microscopy of LPS and LPS-Hb mixtures

Each LPS (1 mg/ml in pyrogen-free NaCl) was incubated with or without Hb (10 mg/ml) for 18 h at 37°C. Negative staining was performed with 2% potassium phosphotungstic acid at pH 6.5 on parlodion-filmed, carbon-coated grids.[12] Briefly, a drop of LPS, LPS/Hb mixture, or Hb was placed on the grid for approximately 30 s. The drop was then drawn off with a Pasteur pipet and excessive material was removed from the grid surface with Whatman #50 filter paper. A drop of 2% phosphotungstic acid was immediately added and, after 15 s contact time, excessive fluid was again removed and the grid allowed to air dry. Grids were examined and photographed in a Siemens 101 electron microscope (Siemens Corp., Medical/Industrial Groups, Iselin, NY, USA) at 80 kV.

Turbidity assay

LPS disaggregation was monitored by sequential turbidity measurements. Turbidity was quantified by absorbance at 700 nm (a wavelength at which there was no contribution from Hb) measured in a DU 7400 UV/VIS scanning spectrophotometer (Beckman Instruments, Inc., Fullerton, CA, USA).

RESULTS AND DISCUSSION

The present study provides a characterization at the ultrastructural level of the LPS disaggregation induced by Hb. We studied two rough LPS preparations that were visibly turbid before the addition of Hb (*S. minnesota* and *E. coli* Re LPS), and a smooth LPS (*E. coli* 055:B5) which was visually clear. The rough *S. minnesota* 595 LPS existed in a number of large, highly aggregated forms, including linear and branched ribbon-like structures with ribbon diameters of approximately 10–20 nm, large mesh-like structures and irregular membrane-like masses, and smaller discoidal structures (Fig. 1). The largest dimensions of many of the highly aggregated particles were in excess of 1 μm. Prolonged sonication of the

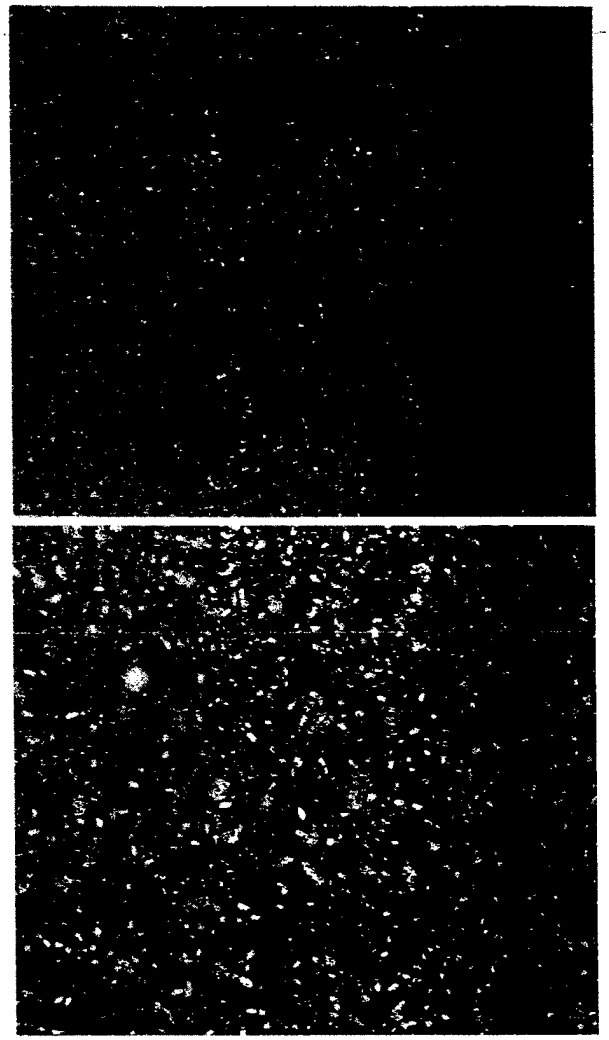

Fig. 2 Top Hb alone, consisting of uniform particles ~5.5 nm (×180,000). **Bottom** Hb plus *S. minnesota* (Re) 595 LPS, following incubation at 37°C for 18 h, showing disaggregation of LPS into small disc (arrow) and lens-shaped (arrow head) particles of 5–20 nm (×180,000). None of the ribbon-like, mesh-like or membrane-like structures of LPS remain after incubation with Hb. Very small Hb-LPS complexes are not distinguishable from Hb alone.

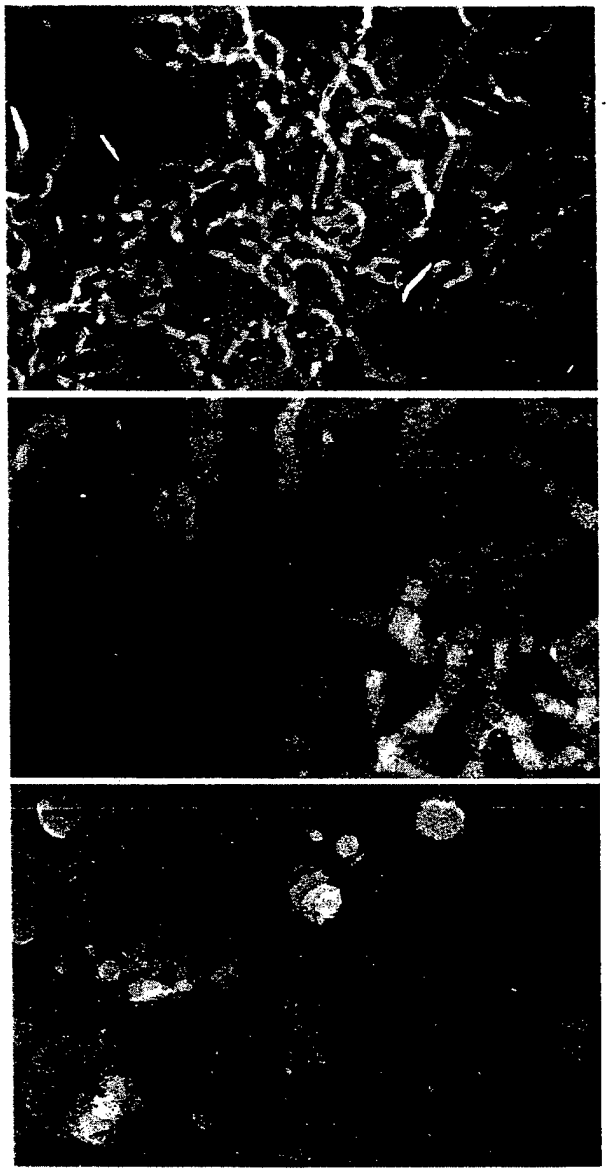

Fig. 3 Top Highly aggregated *E. coli* (Re) LPS demonstrating primarily ribbon-like structures (arrows) continuous with sheet-like regions (open arrows) (×60,000). **Middle** High-power image (×180,000) of the ribbon-like LPS structures. **Bottom** Hb plus *E. coli* (Re) LPS, following incubation at 37°C for 18 h, showing disaggregation of LPS into heterogeneous membrane-like particles of 80–200 nm (×180,000). None of the ribbon-like structures of *E. coli* (Re) LPS alone are present.

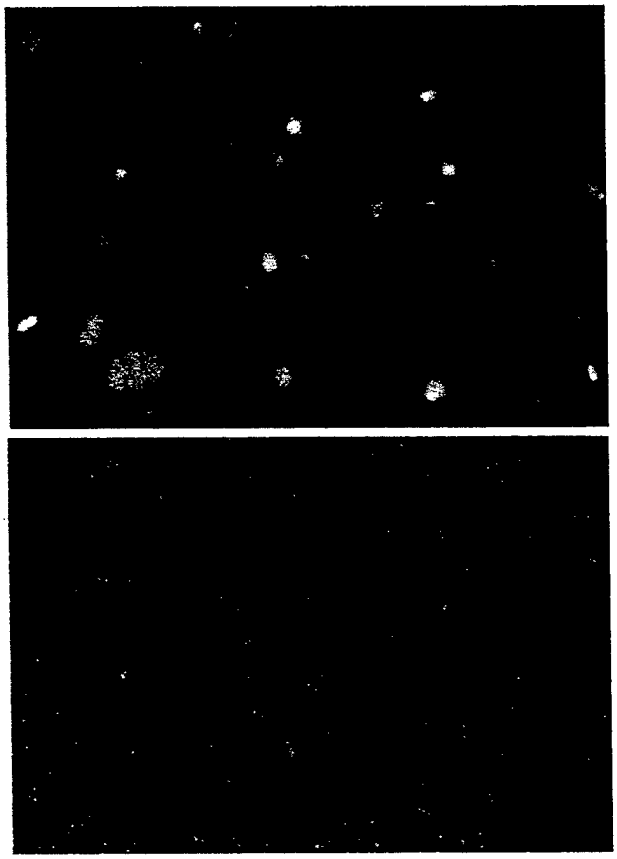

Fig. 4 Top Smooth *E. coli* 055:B5 LPS consists of heterogeneous particles, 10–100 nm (×180,000). **Bottom** Hb plus smooth *E. coli* 055:B5 LPS, following incubation at 37°C for 18 h, showing loss of most of the largest LPS particles.

LPS favored the ribbon-like structures. Hb alone (Fig. 2, top) appeared as small, uniform round particles of ~5.5 nm. After incubation of *S. minnesota* 595 LPS with Hb for 18 h (Fig. 2, bottom), none of the large mesh-like masses or ribbon-like structures remained, and the resultant LPS particles were small disc to lens-shaped particles of 5–20 nm, as well as 5.5 nm particles indistinguishable from Hb alone. *E. coli* (Re) LPS alone was composed predominantly of large ribbon-like structures and occasionally mesh-like structures (Fig. 3, top & middle), similar to the morphology of *S. minnesota* 595 LPS. After incubation with Hb, none of the ribbon-like structures remained, and the LPS appeared as circular membrane-like sheets of 80–200 nm (Fig. 3, bottom). Corresponding to the disappearance of the large *S. minnesota* 595 and *E. coli* LPS structures, each of the mixtures of rough LPS and Hb, initially turbid, became visually clear after incubation. Smooth *E. coli* 055:B5 LPS alone was visibly clear, and was composed of heterogeneous round and oblong particles of 10–100 nm (Fig. 4, top), with none of the extremely large aggregates that were observed with the rough LPS preparations. After incubation of *E. coli* 055:B5 LPS with Hb, most of the large and most irregular LPS structures had become disaggregated (Fig. 4, bottom). As has been shown previously, biological activities of both *S. minnesota* 595 and *E. coli* 055:B5 LPS, as assessed by the *Limulus* amebocyte lysate (LAL) test, were greatly increased in the presence of Hb (data not shown).

Because the rough LPS structures were sufficiently large to scatter light, we were able to monitor Hb-induced disaggregation of the rough LPS ribbon-like and mesh-like structures by spectroscopic measurement of turbidity (Fig. 5). In a representative experiment there was progressive decrease in turbidity of *S. minnesota* 595 LPS after addition of Hb, with a 50% reduction in turbidity after approximately 5 h. In 4 independent experiments, the time for complete loss of opacity of *S. minnesota* LPS ranged from 8–30 h after addition of Hb. Although this process is relatively slow, in vivo experiments in our laboratory have demonstrated[10] that Hb infusion in mice enhances LPS-related mortality even when administered 12 h after LPS, suggesting that prolonged LPS-Hb interactions may be significant. The effect of Hb on LPS is of additional interest because this time is considerably after the LPS-induced tumor necrosis factor (TNF) activity spike. Therefore, Hb may be able to increase LPS biological activity in vivo late in the course of endotoxemia by mechanisms possibly including LPS disaggregation.

The heterogeneity of structures (e.g. ribbons, mesh-like structures, and membrane-like particles) exhibited by *S. minnesota* and *E. coli* LPS was similar to those described previously for enteric LPS.[13–16] LPS from *Actinobacillus pleuropneumoniae*, a non-enteric animal pathogen, also consists of ribbons and vesicles;[17] interestingly, this LPS has been shown to be capable of binding hemoglobin.[18] We did not observe trilaminar structures, as have been described previously for *S. typhimurium*.[13] In

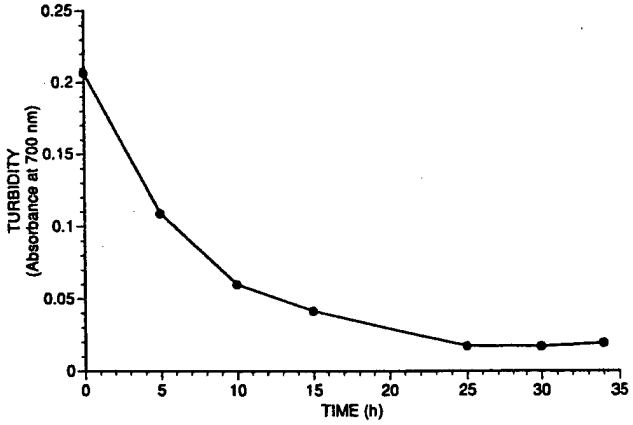

Fig. 5 Hb-induced disaggregation of LPS. *S. minnesota* 595 LPS in water was incubated with HbA_0 (each 1 mg/ml, final concentration), and changes in turbidity were monitored by sequential absorbance measurements at 700 nm.

our present study, Hb induced disaggregation of very large LPS structures into small discoidal and membrane-like particles. Under the experimental conditions utilized (10-fold excess of Hb by weight, corresponding to a 1:2–3 molar ratio of Hb to LPS based on a typical monomer molecular mass of 2,500 Da for rough LPS), all of the LPS had become disaggregated. It is likely that some of the Hb–LPS complexes were composed of a single monomeric rough LPS bound to Hb, producing particles that would not likely be ultrastructurally distinguishable from unbound Hb due to their very small size. Ultrastructural changes induced in the smooth *E. coli* 055:B5 LPS were not nearly as evident as with the rough LPSs. This was a surprising finding since a similar smooth *E. coli* LPS (026:B6) is known to have increased filterability through 300 kDa and 100 kDa membranes when complexed to Hb.[6] However, it is possible that much of this LPS in the Hb-LPS complexes was of sufficiently small size to be indistinguishable from unbound Hb in the photomicrographs.

The relationship between LPS aggregate size in Hb–LPS complexes and LPS biological activity is of interest considering the known affinity of LPS for a variety of LPS-binding proteins that are known to either facilitate[19–23] or inhibit[24–26] LPS biological activity. In this and in our previous studies,[6–8,27] Hb clearly enhances LPS biological activity. Polymyxin B, which is known to bind to LPS with very high affinity and neutralize LPS biological activity, also has been reported to disaggregate LPS when examined by electron microscopy,[26] although other investigators demonstrated with chromatographic and centrifugation studies that LPS-polymyxin B complexes were of greater molecular weight than LPS alone.[28] Human albumin, another known LPS-binding protein,[29,30] has no effect on *S. minnesota* LPS turbidity and has little or no effect on LPS activity.[7] Therefore, it is possible that disaggregated LPS in Hb–LPS complexes is more efficiently presented to effector mechanisms (e.g. plasma coagulation proteins, and LPS receptors on blood cells and endothelial cells). In support of this possible mechanism, we have recently demonstrated that the binding of LPS to endothelial cells is increased when LPS is bound to Hb.[31] Therefore, it is likely that the mechanism of LPS presentation by Hb is different from that with lipopolysaccharide binding protein (LBP), in which monomer units of LPS are transferred to soluble CD14 from LPS–LBP complexes without disaggregation of LPS.[32]

An additional potential mechanism for the enhancement of LPS biological activity by Hb is suggested by studies of the relative potencies of monomeric vs aggregated rough *E. coli* LPS, which demonstrated that monomeric LPS was as much as 1000-fold more active than aggregated LPS in in vitro assays.[33] It was proposed that time-dependent generation of small concentrations of monomeric LPS (0.01–0.6% of the total LPS) was an important variable in assays of LPS activity. Therefore, it is possible that more free monomeric LPS might be generated from low molecular weight Hb-LPS complexes than from highly aggregated LPS alone, and that the mechanism of Hb enhancement of LPS biological activity might involve facilitation of monomer LPS production. Biophysical studies of the 3-dimensional structures exhibited by aggregated LPS have demonstrated a diverse range of nonlamellar conformations[34,35] which would be expected to contain much of the LPS buried within the macromolecular complexes. We postulate here that one mechanism by which Hb-induced disaggregation of LPS macromolecular aggregates results in greatly increased LPS biological activity is to expose many of the toxic domains of LPS (i.e. lipid A) that are cryptic in highly aggregated LPS.

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Hemoglobin Enhances the Binding of Bacterial Endotoxin to Human Endothelial Cells

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Summary

Human endothelial cells, when incubated with bacterial endotoxin (lipopolysaccharide, LPS), modify their surface in association with prominent production of procoagulant tissue factor (TF) activity. This deleterious biological effect of LPS has been shown previously to be enhanced approximately 10-fold by the presence of hemoglobin (Hb), a recently recognized LPS binding protein that causes disaggregation of LPS and increases the biological activity of LPS in a number of in vitro assays. The present study was performed to test the hypothesis that Hb enhances the LPS-induced procoagulant activity of human umbilical vein endothelial cells (HUVEC) by increasing LPS binding to the cells. The binding of ^{3}H-LPS to HUVEC was determined in the absence or presence of Hb or two other known LPS-binding proteins, human serum albumin (HSA) and IgG. LPS binding was substantially increased in the presence of Hb, in a Hb concentration-dependent manner, but was not increased by HSA or IgG. Hb enhancement of LPS binding was observed in serum-free medium, indicating that there was no additional requirement for any of the serum factors known to participate in the interaction of LPS with cells (e.g., lipopolysaccharide (LPS)-binding protein (LBP) and soluble CD14 (sCD14)). Hb enhancement of LPS binding also was observed in the more physiologic condition of 100% plasma. LPS-induced TF activity was stimulated by Hb, but not by HSA or IgG. In serum-free medium, TF activity was not stimulated under any of the conditions tested. Ultrafiltration of LPS was dramatically increased after incubation with Hb but not with HSA or IgG, suggesting that LPS disaggregation by Hb was responsible for the enhanced binding of LPS to HUVEC and the subsequent stimulation of TF activity.

Introduction

Human endothelial cells are known to interact with bacterial endotoxin (lipopolysaccharide, LPS), a highly toxic component of the cell wall of gram negative bacteria which is commonly present in the blood during gram-negative bacterial sepsis. A major consequence of this interaction is the transformation of the endothelium from an anticoagulant surface to a procoagulant surface (1, 2). Recent studies from our laboratory have demonstrated that the interaction of LPS with endothelial cells is modulated by human hemoglobin (Hb) (3). Specifically, deleterious induction of endothelial cell tissue factor (TF) by LPS is dramatically enhanced (typically 10-fold) when LPS is in the presence of Hb. We have recently demonstrated that Hb is an LPS binding protein which causes LPS disaggregation (4, 5), and other pathological effects of LPS not involving the endothelium similarly, have been shown to be increased by Hb, e.g., activation of Limulus amebocyte lysate (4-6) and induction of mononuclear cell procoagulant activity (6). In vivo, LPS and Hb have been shown to produce synergistic toxicity in rabbits (7), and ongoing studies in our laboratory have demonstrated synergistic mortality produced by the combination of intraperitoneal LPS and intravenous Hb in mice (unpublished data). The interaction of Hb with LPS is of considerable current interest because of the ongoing efforts to develop cell-free Hb-based oxygen carriers for resuscitation (8) which can be administered after traumatic injury in which endotoxemia frequently will be present. Toxicity of infused Hb is of considerable concern (9), and the enhancement by Hb of the responses of endothelial cells to LPS would be of particular concern for resuscitation during sepsis.

The interaction of LPS with cells characteristically involves both soluble LPS binding proteins and cellular receptors (10-13). Since Hb binds and disaggregates LPS, it is possible that Hb results in more efficient presentation of LPS to the endothelium, thereby causing an augmented endothelial cell response to LPS. The present studies were conducted to determine whether a contributory mechanism for Hb enhancement of LPS-induced endothelial cell TF activity might involve a stimulation of LPS binding to the endothelial cells.

Methods

Reagents

Human serum albumin (HSA) (25%, for injection) was purchased from Nybcen (New York, NY), human immunoglobulin (IgG) (185 mg/ml) from Armour Pharmaceutical Co. (Kankakee, IL), and hirudin from Sigma Chemical Co. (St. Louis, MO). Sterile tissue culture plasticware was obtained from Becton Dickinson (Mountain View, CA).

Hemoglobin

Human crosslinked cell-free hemoglobin (αα Hb), covalently crosslinked between the two α chains with bis(3,5-dibromosalicyl) fumarate as described previously (14), was provided by collaborators at the Blood Research Detachment, Walter Reed Army Institute of Research (WRAIR), Washington, DC. The Hb preparation contained less than 0.25 EU/ml endotoxin (referenced to E. coli lipopolysaccharide B, O55:B5, Difco Laboratories, Detroit, MI), as determined by the Limulus amebocyte lysate test (15), and did not contain demonstrable erythrocyte stroma, as demonstrated by phosphorus analysis and reverse phase high pressure liquid chromatography. Hb concentrations were determined spectrophotometrically.

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Endotoxin

E. coli K12 LPS, biosynthetically labeled with ^3H (1.6×10^6 dpm/μg), was obtained from List Biological Laboratories, Inc., Campbell, CA, and E. coli O55:B5 LPS from Difco Laboratories (Detroit, MI).

HUVEC

Human umbilical vein endothelial cells (HUVEC) and endothelial cell culture medium containing 2% fetal bovine serum, 10 ng/ml epidermal growth factor (EGF) and 1 ng/ml hydrocortisone were purchased from Clonetics (San Diego, CA). The medium was supplemented with additional bovine calf serum (Cosmic Calf, HyClone Laboratories, Logan, UT) to generate 4% total serum. HUVEC were plated in 24-well microtiter plates (Nunclon, Applied Scientific, South San Francisco, CA) in 0.5 ml media at a seeding density of 3,500 cells/cm^2 and grown to confluency at 37° C and 5% CO_2. HUVEC were utilized at less than 6 passages. For selected experiments, the standard medium was removed from confluent HUVEC monolayers; the cells then were washed 3 times with medium not containing the bovine serum and subsequently equilibrated for 1 h in the serum-free medium before performance of binding assays.

LPS Binding Assay

^3H-LPS was incubated with Hb, HSA or IgG for 30 min at 37° C, and then the ^3H-LPS or ^3H-LPS-protein mixtures were added to confluent HUVEC monolayers, in either serum-free or complete media. In typical experiments, 50 μl of sample containing 50,000-200,000 cpm (^3H-LPS, 0.05-0.2 μg) and 100 mg/ml Hb, HSA or IgG was added per well containing 0.5 ml media. After incubation for 30 min at 37° C, the medium was removed and the monolayers were washed 3 times with fresh medium. The HUVEC were then solubilized in 0.7% NaOH, and the bound ^3H-LPS was quantified by scintillation counting, after samples were diluted 10-fold in fluor (Formula A-989, NEN Research Products, Boston, MA), in a Tracor Analytic Liquid Scintillation System (Tracor Analytic, Elk Grove Village, IL). In selected experiments, binding assays were performed in hirudin anticoagulated plasma (which avoided potential effects of calcium chelators or heparin on the HUVEC) in the absence of medium. In additional experiments, the HUVEC were pre-cooled, and LPS binding assays were performed at 4° C.

Assays of HUVEC Integrity

HUVEC confluent monolayers were incubated overnight with 10^5 cpm ^3H-Leucine (DuPont NEN, Wilmington, DE), and then washed 3 times with fresh medium. With the cells at this point in non-radioactive medium, E. coli O55:B5 LPS (at final concentrations of 1-10 μg/ml) or preincubated mixtures of LPS and 10 mg/ml Hb, HSA or IgG (prepared as above) were then added to the wells. After 30 min at 37° C, the medium was removed and ^3H released from the cells was quantified by scintillation counting. In selected experiments, HUVEC exposed to LPS or LPS-protein mixtures also were assayed for viability by trypan blue exclusion.

TF Procoagulant Assay

TF activity was quantified with a plasma recalcification assay as described previously (3). Briefly, confluent HUVEC monolayers in 24-well plates were incubated for 1 h at 37° C with E. coli K12 LPS alone or mixtures of LPS and Hb, HSA or IgG, as described above for the LPS binding assay. In these experiments, the final LPS concentrations were 0.2 μg/ml, and the final Hb, HSA or IgG concentrations were 10 mg/ml. Wells were then washed with medium (x3) and the HUVEC were freeze-thawed twice and finally sonicated in 0.5 ml phosphate buffered saline for 2 min at room temperature. 150 μl from each well was transferred to a 96-well microtiter plate, and 50 μl normal human citrated plasma followed by 20 μl calcium chloride (25 mM) were then added to each well. Development of turbidity (clot formation) was measured at 340 nm in a 37° C temperature-controlled plate reader (Kinetic-QLC, Whittaker Bioproducts Inc., Walkersville, MD). Increased TF activity was demonstrated by a faster rate of increase in absorbance at 340 nm and a shortened lag period.

Ultrafiltration

Ultrafiltrations were performed using XM 300 (300 kDa cut-off) ultrafiltration membranes (Amicon Division, W.R. Grace, Danvers, MA). ^3H-LPS was incubated with Hb, HSA or IgG for 30 min at 37° C, and then LPS or LPS-protein mixtures were filtered manually with a 3 ml syringe (according to the directions of the filter manufacturers) at room temperature. ^3H-LPS in the filtrates was quantified by scintillation counting. For samples containing Hb, quenching of ^3H-LPS was reversed as follows: 0.1 ml aliquots of fractions were

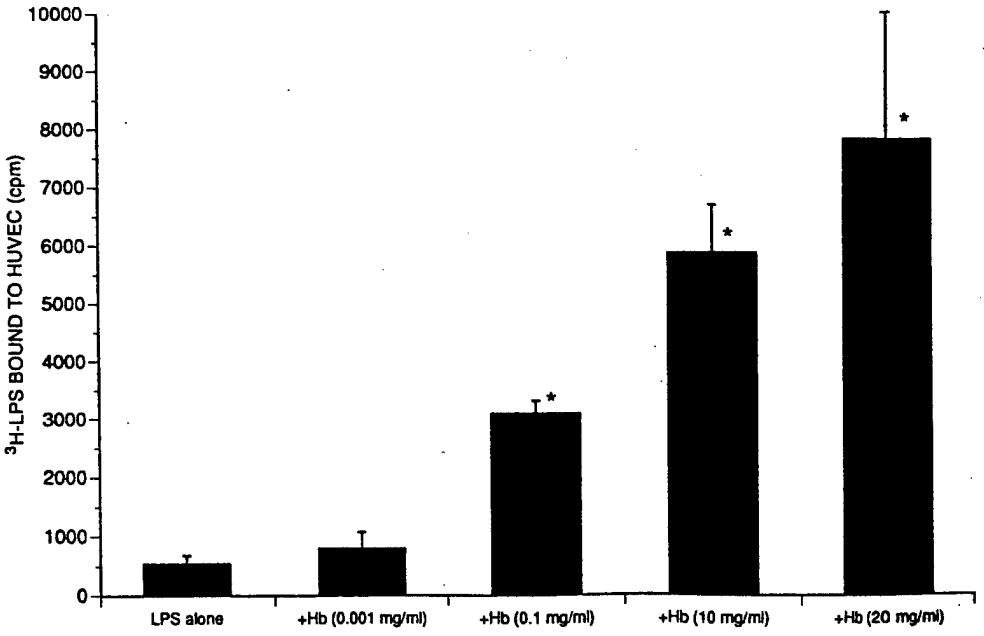

Fig. 1 Effect of Hb on binding of LPS to HUVEC. ^3H-LPS was preincubated with various concentration of Hb, and ^3H-LPS alone or the LPS-Hb mixtures then were added to HUVEC in medium containing 4% bovine serum. After 30 min, bound LPS was determined by scintillation counting. Binding assays were performed in two replicate wells per condition. Means and ranges are presented.
*p <.01 increase vs. LPS alone (Student's T-test)

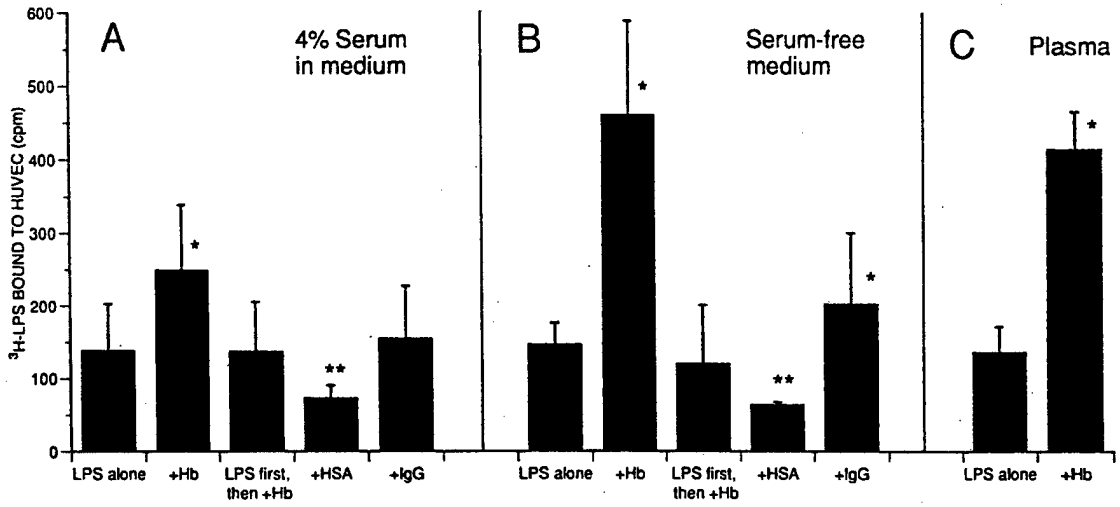

Fig. 2 Effect of proteins on binding of LPS to HUVEC. ^{3}H-LPS was preincubated with Hb, HSA or IgG (each 10 mg/ml, final concentration), and ^{3}H-LPS alone or the LPS-protein mixtures then were added to HUVEC. After 30 min, bound LPS was determined by scintillation counting. In some wells, ^{3}H-LPS was added to the medium first, followed by the subsequent addition of Hb 5 min later. Binding assays were performed with 6 replicate wells in complete medium containing 4% bovine serum (A), in serum-free medium (B) or in 100% plasma (anticoagulated with hirudin) in the absence of medium. Means ± SD are presented.
*p <.01 increase vs. LPS alone (Student's T-test). **p <.01 decrease vs. LPS only

diluted ten-fold in water (to 1 ml final volume), and 1 ml Solvable (NEN Research Products, Boston, MA) was added. These mixtures were incubated at 60° C for one hr, and then 0.3 ml 25% H_2O_2 was added. After 30 min additional incubation at room temperature, samples were pale yellow in color, and could be analyzed for radioactivity. Recovery of spiked radioisotope in preliminary experiments to determine the effectiveness of the decolorizing procedure demonstrated >98% detection of previously added radioactivity.

Results

Effect of Hb on LPS Binding to HUVEC

^{3}H-LPS binding to HUVEC was reproducibly enhanced when LPS had been preincubated with Hb (final Hb concentration of 10 mg/ml). Mean enhancement by 10 mg/ml Hb in 10 independent experiments was 4.1-fold (median 5.3-fold; range, 1.8-10.8 fold). In the experiment which demonstrated the greatest stimulation by Hb (10.8-fold), the enhancement of ^{3}H-LPS binding to HUVEC was shown to be dependent upon Hb concentration between 0.001 mg/ml and 20 mg/ml Hb (Fig. 1). To determine whether LPS binding was associated with cell damage, non-radiolabeled E. coli LPS was incubated with HUVEC preloaded with ^{3}H-Leucine. Neither LPS alone nor Hb-LPS mixtures caused any increase in released radioactivity, and cell viability as assessed by trypan blue exclusion was >99% for all conditions (data not shown).

LPS Binding to HUVEC: Comparison of Hb with Other LPS Binding Proteins

Hb, HSA or IgG were preincubated with ^{3}H-LPS to form complexes prior to addition to HUVEC, and then LPS binding was determined. Whereas Hb enhanced LPS binding to HUVEC (nearly 2-fold in this experiment), HSA slightly inhibited LPS binding and IgG had no effect (Fig. 2A). When LPS was added to the medium first, and Hb was subsequently added approximately 5 min later (i.e., there was no preincubation to form Hb-LPS complexes), there was no effect of Hb on LPS binding. This suggested that LPS may have been rapidly complexed to high affinity LPS binding factors in the serum component of the medium, and that subsequent addition of Hb had no effect on this distribution. Binding experiments were also performed at 4° C rather than 37° C in order to minimize LPS uptake. Binding of LPS alone at 4° C was equivalent to LPS binding at 37° C, and enhancement by Hb was observed (4.2-fold). To investigate the potential role of serum factors in the enhancement by Hb of LPS binding, binding experiments were performed using serum-free medium. Hb substantially stimulated LPS binding (approximately 3-fold), indicating that no other protein factors were required for this effect of Hb (Fig. 2B). In serum-free medium HSA slightly inhibited binding, and IgG slightly increased LPS binding. Therefore, the results of LPS binding experiments performed in complete (4% serum) and serum-free media were qualitatively similar. Hb also enhanced LPS binding in the presence of 100% plasma (with no medium present during the 30-min binding incubation) (Fig. 2C). With these conditions, the slight inhibition of LPS binding that had been observed with addition of HSA to medium (Fig. 2A and B) was not observed despite approximately 4-fold greater concentration of albumin in the incubation. It is possible that the higher concentrations of other LPS binding proteins of high affinity (e.g., LBP) in 100% plasma relative to their concentration in 4% serum prevent any possible LPS-albumin interaction, accounting for the lack of observable HSA inhibition of LPS binding.

TF Production by HUVEC: Comparison of the Effect of Hb with Other LPS Binding Proteins

Since Hb enhanced LPS binding to HUVEC, and Hb was known from previous studies to form complexes with LPS (4) and to stimulate LPS-induced TF production (3), it seemed likely that enhanced LPS binding to HUVEC by Hb-LPS complexes provided the mechanism for increased stimulation of the TF response. HSA and IgG are also recognized as LPS binding proteins, but in contrast to Hb they did not increase LPS binding to HUVEC, as described above. Neither HSA-LPS complexes nor IgG-LPS complexes resulted in appreciably different levels of HUVEC TF activity than did LPS alone, whereas Hb-LPS

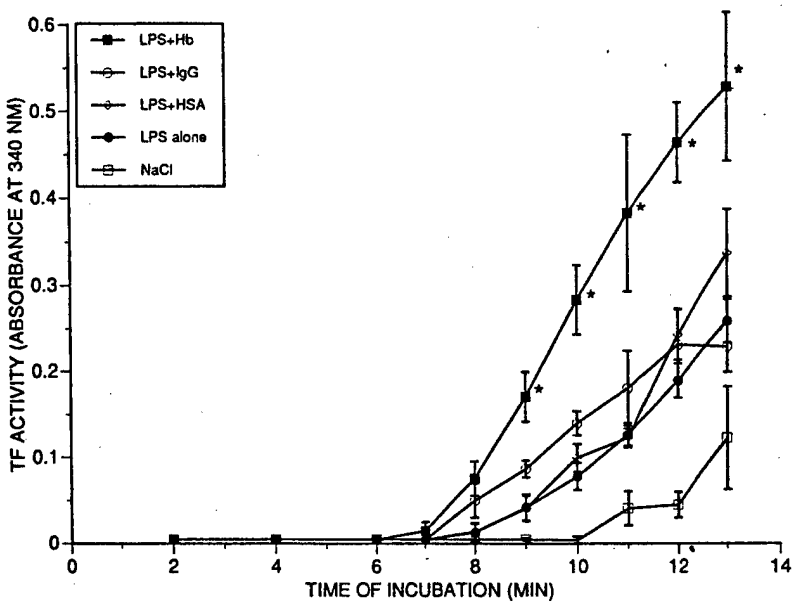

Fig. 3 Tissue factor (TF) procoagulant activity. LPS was preincubated with Hb, HSA or IgG, and LPS alone or the LPS-protein mixtures then were added to HUVEC in medium containing 4% bovine serum. After 1 h, the HUVEC were washed, freeze-thawed and sonicated, and citrated plasma and calcium were added as described in Methods. TF activity was assessed by the rate of increase in absorbance at 340 nm. Mean ± SD absorbances from 12 replicate wells are presented.
*p <.01 increase vs. LPS alone (Student's T-test)

complexes were stimulatory (Fig. 3). Interestingly, whereas LPS binding to HUVEC in both the absence and presence of Hb did not require serum factors (above), neither LPS alone nor LPS-Hb complexes induced TF from HUVEC when the experiments were conducted in serum-free medium (data not shown).

Disaggregation of LPS by Hb

Hb is known to result in LPS disaggregation, and it has been postulated that LPS binding and disaggregation by Hb is responsible for the generation of LPS that is more biologically active than highly aggregated unbound LPS (4,5). Therefore, experiments were conducted to assess whether the differences in LPS binding to HUVEC and subsequent TF production that resulted from pre-incubation of LPS with Hb, HSA or IgG might be related to differential abilities of these LPS-binding proteins to cause LPS disaggregation. Disaggregation of radiolabeled LPS was evaluated by ultrafiltration through 300 kDa cut-off membranes. Whereas LPS alone was highly aggregated and little of this LPS was filterable (5.5%), approximately 6-fold more LPS from LPS-Hb mixtures was filterable (31%). In contrast, HSA did not increase the filterability of LPS (6% filtered) and IgG slightly reduced filterability (0.3%), indicating that LPS was not disaggregated in these mixtures.

Discussion

Modulation of LPS interactions with the vascular endothelium is of particular interest because of the critical role of the endothelium in the pathophysiology of bacterial sepsis. Transformation of the endothelial lining from an anticoagulant surface to a procoagulant surface contributes to the development of diffuse coagulopathy during sepsis. With the development of Hb-based red blood cell substitutes and their anticipated use in patients who may have concomitant endotoxemia, interactions between Hb and LPS have potentially important clinical relevance. Recently, Hb has been shown to bind LPS and cause its disaggregation, and LPS in the resultant complexes has been demonstrated to have enhanced biological activity in several model systems (3-6). The interaction of LPS with HUVEC is one such model system, and the present studies were performed to investigate the mechanism by which Hb enhances the procoagulant (TF) response of HUVEC to LPS (3). It has been proposed that Hb exerts its enhancement effect on LPS biological activity by increasing LPS solubility and improving the presentation of LPS to effector cells and proteins (3,5). LPS binding to endothelial cells is the first step in LPS activation of the endothelial surface (11), suggesting that a possible mechanism for Hb enhancement of LPS biological activity could be the presentation of more LPS available for cell binding. Investigation of this potential mechanism was the rationale for the present studies.

The present work demonstrated that there is a quantitative increase in LPS binding to HUVEC when LPS has been complexed with Hb prior to its interaction with the cells. There was no such effect of two other LPS binding proteins, HSA or IgG, and the LPS disaggregation and increased LPS biological activity (TF production) observed after incubation of LPS with Hb was not observed with HSA and IgG. Together, these studies strongly suggest an association between LPS disaggregation, increased efficiency of LPS binding to HUVEC, and enhanced induction by LPS of HUVEC procoagulant activity. Since LPS binding to HUVEC also was increased by Hb when experiments were performed in 100% plasma, it appears that the presence of physiologic concentrations of high affinity LPS binding proteins (e.g., LBP) does not interfere with the Hb enhancement effect. This suggests that the in vitro findings presented in this study may also be relevant in vivo under actual physiologic conditions of endotoxemia. Since Hb alone has no effect on the minimal TF activity of HUVEC in the absence of LPS (3), and since the magnitude of the stimulation of HUVEC TF activity is directly related to LPS concentration (3), it is reasonable to conclude that the increased binding of LPS to the cells contributes to the enhanced cellular response. It is not possible, however, to rule out a potential additional contribution of Hb to the augmentation of LPS signal transduction or to otherwise contribute to cell activation; i.e., Hb might also present LPS to cellular receptors such that there would be more efficient signal transduction independent of quantitatively increased LPS binding to HUVEC.

The present studies showed for the first time that LPS could bind to HUVEC in serum-free medium and that, under this condition, Hb was capable of increasing LPS binding to HUVEC. However, TF production was not stimulated in the absence of serum factors. Optimal LPS

stimulation of the endothelium requires that the serum factor lipopolysaccharide (LPS)-binding protein (LBP) facilitate the binding of LPS to another serum factor soluble, CD14 (sCD14) (11,16). LPS-sCD14 complexes in turn interact with an as yet uncharacterized cellular receptor to promote the direct activation of HUVEC by LPS (11,16). In serum-free media there was no source of LBP or sCD14, and although LPS binding to HUVEC was supported by Hb, Hb was not able to substitute for LBP and sCD14 in production of the TF biological response. Therefore, it appears that although Hb-LPS complexes in the absence of serum efficiently bind to HUVEC, they may not bind to the cellular receptor for LPS-sCD14, or alternatively they may interact with the receptor in a manner that does not produce the ultimate response of TF production.

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Hemoglobin Increases Mortality from Bacterial Endotoxin

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Cell-free hemoglobin (Hb) is being developed as an erythrocyte substitute. We have previously demonstrated that cell-free Hb is an endotoxin-binding protein which disaggregates endotoxin and subsequently increases the biological activity of endotoxin in several in vitro assays. Because much of the morbidity and mortality associated with gram-negative bacterial infection is the result of pathophysiologic responses to bacterial lipopolysaccharide (LPS; endotoxin), we studied the effect of Hb on LPS-mediated mortality. Hb infused intravenously into mice before, coincident with, or after intraperitoneal LPS injection substantially increased LPS-related mortality from <5% to 50 to 70% 24 h after administration of LPS and from 50% to 60 to 90% at 48 h. Enhanced mortality was observed over a range of doses of injected LPS. At a given LPS dose, enhancement of mortality was shown to be dependent on the dose of Hb administered. Unmodified native human Hb, α-α-cross-linked human Hb, and β-β-cross-linked human or bovine Hb all were shown to enhance LPS-mediated mortality. Depressed reticuloendothelial cell function may have contributed to the enhanced mortality from LPS in the presence of Hb. Therefore, Hb-based blood substitutes, which are currently undergoing clinical trials, may intensify the potentially fatal effects of the sepsis syndrome in patients with trauma, infection, or hypotension who receive Hb for erythrocyte replacement.

Because of the known risks and limitations of transfused erythrocytes as oxygen carriers (infections, donor-recipient incompatibility, limited shelf life, requirement for refrigerated storage), there is great interest in the development of acellular hemoglobin (Hb)-based oxygen carriers (cell-free Hb). Both the Food and Drug Administration and the military have recognized the need for high-priority development of a stable, infectious-agent-free erythrocyte substitute. However, cell-free Hb exhibits specific biochemical properties that are either intrinsically toxic or capable of eliciting deleterious host responses. One worrisome characteristic of Hb is its potential interaction with infectious agents. In particular, it has been well documented that lethality associated with gram-negative infection can be enhanced by Hb (7, 8, 18, 19), and elucidation of the mechanism(s) that contributes to this deleterious effect of Hb is presently of major interest. In some studies, increased bacterial toxicity in the presence of Hb has been attributed to stimulation of bacterial growth by iron (7, 8), although this result has not been uniformly reported (16, 17). In other studies, inhibition by Hb of leukocyte chemotaxis, phagocytosis, and killing of bacteria has been demonstrated (9, 10). Depression of reticuloendothelial cell system function also has been implicated in the enhanced mortality from bacterial infections that occurs in the presence of hemoglobinemia due to erythrocyte hemolysis (14, 15). Finally, a decade ago, synergistic toxicity was observed following administration of Hb and bacterial endotoxin (lipopolysaccharide [LPS]) (30), the cell wall component of gram-negative bacteria responsible for much of the toxicity produced by bacterial sepsis. In that report, lethality in rabbits which simultaneously received both intravenous stroma-free Hb and *Salmonella enteritidis* LPS was significantly greater than lethality observed after LPS alone (30).

Recently, there has been renewed interest in the potentially important role of deleterious LPS-Hb interactions. Our laboratory has demonstrated that Hb forms stable complexes with a wide variety of LPSs and that LPS macromolecular particles become disaggregated after binding to Hb (11, 12). In a series of in vitro studies, Hb was shown to enhance the ability of LPS to activate coagulation (11, 25), stimulate procoagulant activity from peripheral blood mononuclear cells (25), and stimulate production of procoagulant activity by cultured human endothelial cells (23). LPS binding to endothelial cells was increased when LPS was complexed with Hb (24), suggesting a possible mechanism for the enhanced cellular response to LPS-Hb complexes compared to LPS alone.

Because these in vitro data suggested potentially substantial pathological consequences of the LPS-Hb interaction, we wished to determine whether coexistent hemoglobinemia and endotoxemia were deleterious in vivo. For these studies, we chose a murine model system frequently used for investigations of sepsis and bacteremia. To mimic a common clinical scenario produced by sepsis resulting from trauma to the gastrointestinal tract or gut ischemia, LPS was injected intraperitoneally into mice, creating prolonged endotoxemia as is characteristic of clinical peritonitis. We used *Escherichia coli* LPS because of the frequent occurrence of *E. coli* sepsis and because of our extensive characterization of LPS-Hb complex formation with use of this LPS (11, 12). Experiments were conducted both with purified native human Hb (HbA_0) and with cross-linked (stabilized) human or bovine Hb.

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MATERIALS AND METHODS

Animals. Female Swiss Webster and C3H/HeJ mice (28 to 32 g) were purchased from Simonsen (Gilroy, Calif.). In conducting research using animals, we adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (5a).

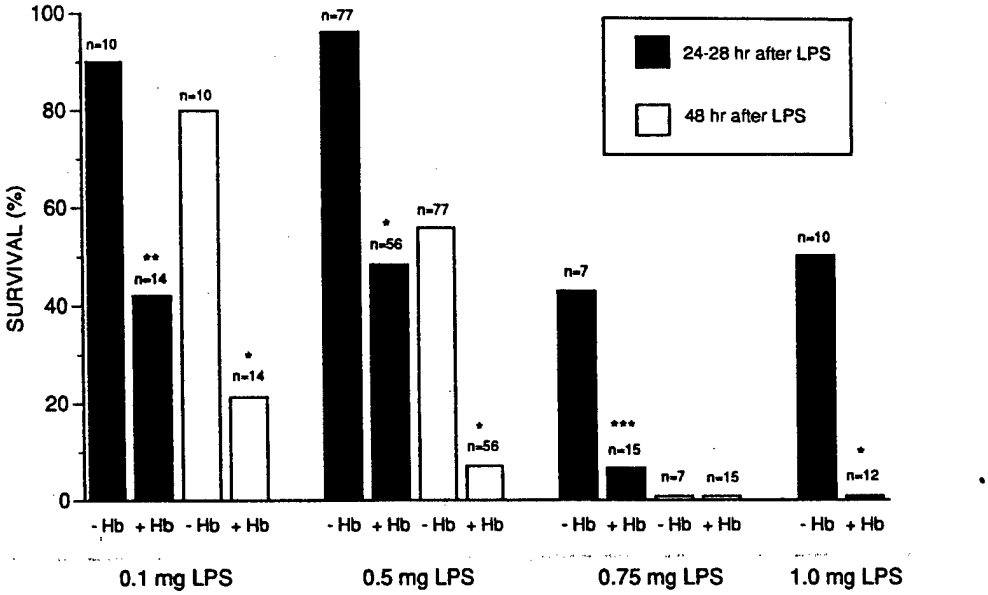

FIG. 1. LPS lethality in mice, at various LPS doses, in the absence (−Hb) and presence (+Hb) of αα-Hb. Swiss Webster female mice (28 to 32 g) were injected intraperitoneally with 0.1, 0.5, 0.75, or 1.0 mg of LPS (*E. coli* O55:B5 LPS, in sterile, pyrogen-free saline). Eight to 10 h after LPS injection, mice were infused by tail vein with either 0.6 to 0.8 ml of saline or 0.6 to 0.8 ml αα-Hb in Ringer's acetate (pH 7.4) (60 mg/mouse), prepared as described in the text. Survival was monitored at 24 to 28 and 48 h after LPS injection. *n*, number of mice in each group. *, $P < 0.01$, +Hb versus −Hb; **, $P = 0.03$, +Hb versus −Hb; ***, $P = 0.07$, +Hb versus −Hb (Fisher's exact *P* value).

Hb. Human Hb, α-α-cross-linked (>98% cross-linked between the α99 lysine residues) with bis(3,5-dibromosalicyl) fumarate (31) (ααHb), was provided by collaborators at the Blood Research Detachment, Walter Reed Army Institute of Research, Washington, D.C. Chromatographically purified, unmodified human HbA_0 (5) also was provided by collaborators at Walter Reed Army Institute of Research. Hb solutions were in Ringer's acetate (pH 7.4), contained <5% methemoglobin, and were sterile and essentially endotoxin free (<100 pg/ml). They were stroma free as determined by phosphate analysis for stromal lipid. Three β-β-cross-linked Hb (ββHb) preparations were generously provided by Enrico Bucci (University of Maryland School of Medicine, Baltimore): bovine fumaryl ββHb, cross-linked between the β81 lysine residues in the β cleft as described previously (29); decasebacyl-cross-linked human β-β/α-α-cross-linked Hb (ββ/ααHb), 50% cross-linked between β82 lysine residues and 50% simultaneously cross-linked between β82 lysine residues and between α99 lysine residues as described previously (4); and another bovine ββHb currently being characterized.

HSA. Human serum albumin (HSA) for infusion, in 0.9% NaCl with sodium bicarbonate (pH 7.4), was obtained from Baxter Corp. (Miami, Fla.).

LPS. *Escherichia coli* LPS O55:B5 and *Salmonella typhosa* LPS O901 were obtained from Difco (Detroit, Mich.). Solutions of 1 mg of LPS per ml in sterile, endotoxin-free 0.9% NaCl (Lymphomed, Deerfield, Ill.) were vortexed and briefly sonicated (1 min) prior to use. *E. coli* K-12 LPS, biosynthetically radiolabeled with ^3H, was obtained from List Biological Laboratories, Inc. (Campbell, Calif.).

Mortality experiments. Mice were injected intraperitoneally with LPS (0.6 to 0.8 ml) and infused intravenously by tail vein with Hb (<1 ml in most experiments). In most experiments, Hb was infused intravenously 8 to 10 h after injection of LPS. In selected experiments, Hb was infused either 12 h prior to or coincident with LPS. In control animals which received intraperitoneal LPS only, sterile, endotoxin-free 0.9% NaCl was infused intravenously so that all animals in an experiment received equal volumes of parenteral fluid. Similarly, in control animals which received intravenous Hb only, NaCl was injected intraperitoneally. In a separate set of control experiments, hemin chloride (Sigma, St. Louis, Mo.) in 0.9% NaCl was infused intravenously 10 h following intraperitoneal administration of LPS or NaCl. Mortality was monitored at 24 to 28 h and at 48 h after injection of LPS. Animals were given standard mouse chow and water ad lib throughout the experiments. Care and manipulation of mice were in accordance with institutional guidelines. Experimental protocols were approved by the Sub-Committee for Animal Studies, Veterans Affairs Medical Center, San Francisco, Calif.

Plasma LPS concentrations. Blood for LPS levels was serially obtained from the retro-orbital plexus in EDTA-containing capillary tubes at various time points after LPS injection, and plasma was prepared by centrifugation of the capillary tubes in a serofuge (International Equipment Co., Needham Heights, Mass.). Variable but low levels of LPS contamination (<0.05 to 2.2 ng/ml) in samples obtained by this technique were deemed acceptable, since 10^2- to 10^4-fold-higher peak plasma LPS levels were achieved in the LPS-injected mice. Plasma LPS concentrations were determined with an Endospecy microplate assay kit generously provided by Seikagaku Kogyo Co. (Tokyo, Japan). Plasma samples were diluted in water (typically 1:1,000 to 1:100,000) until LPS concentrations were within the evaluable range and then assayed with the Endospecy *Limulus* amebocyte assay (LAL) reagent, containing amebocyte lysate prepared from *Tachypleus tridentatus*, buffer, and chromogenic substrate. This range of dilutions was sufficient to obviate use of the alkalinization step recommended by the manufacturer to remove inhibitors. Incubations were carried out with an SK601 well reader (Seikagaku), and LPS concentrations were determined by kinetic analyses of absorbances at 405 nm, with standard *E. coli* O111:B4 LPS provided by Seikagaku as the reference.

Plasma LPS concentrations were also determined by using recovery of radiolabeled LPS. In these experiments, mice received an intraperitoneal injection of ^3H-LPS (5×10^5 cpm; 0.3 μg of LPS) with or without 500 μg of unlabeled LPS. Plasma samples were obtained at various time points after LPS injection, and plasma LPS concentrations were determined from ^3H measurements by using a liquid scintillation counting system (Tracor Analytic, Elk Grove Village, Ill.).

Plasma glucose concentrations. Blood for determination of glucose levels was serially obtained from the retro-orbital plexus in EDTA-containing capillary tubes at various time points up to 48 h after LPS injection, with and without intravenous Hb infusion coincident with the LPS injection. In these experiments, all animals were fasted (water ad lib was provided) following LPS injection. Plasma was prepared by centrifugation of the capillary tubes in a serofuge (International Equipment Co.), and glucose concentrations were determined with a Glucose (HK) assay kit (Sigma).

Hb clearance. Blood for measurement of Hb levels was serially obtained from the retro-orbital plexus in similar capillary tubes at various time points after Hb infusion, and plasma was obtained by centrifugation as described above. Plasma samples were diluted 1:15 with 0.9% endotoxin-free saline, and Hb clearance was monitored at 405 nm in a BioWhittaker (Walkersville, Md.) Kinetic-QCL plate reader.

Particulate carbon clearance. Carbon (India ink) was purchased from Baxter Corp. and diluted in sterile, endotoxin-free 0.9% saline such that a subsequent 1:50 dilution had an absorbance of approximately 0.6 at 620 nm. Mice were injected with intraperitoneal NaCl or LPS followed by intravenous NaCl or Hb 10 h later, and after an additional 10 h, carbon solutions were infused intravenously by tail vein. Serial blood samples were obtained from the retro-orbital plexus with EDTA-containing capillary tubes at various time points (0.5 to 30 min) after injection. The samples of whole blood were diluted 1:10 in water to lyse the erythrocytes, and absorbances due to carbon were determined at 620 nm.

Blood cultures. Mice were injected intraperitoneally with LPS and 10 h later received Hb intravenously. Blood samples (~1 ml) were obtained for culture (Bacto Columbia Broth; Difco) by cardiac puncture 26 and 36 h after LPS injection. Cultures were incubated aerobically at 37°C for 30 days and periodically plated on G-C agar medium (Difco).

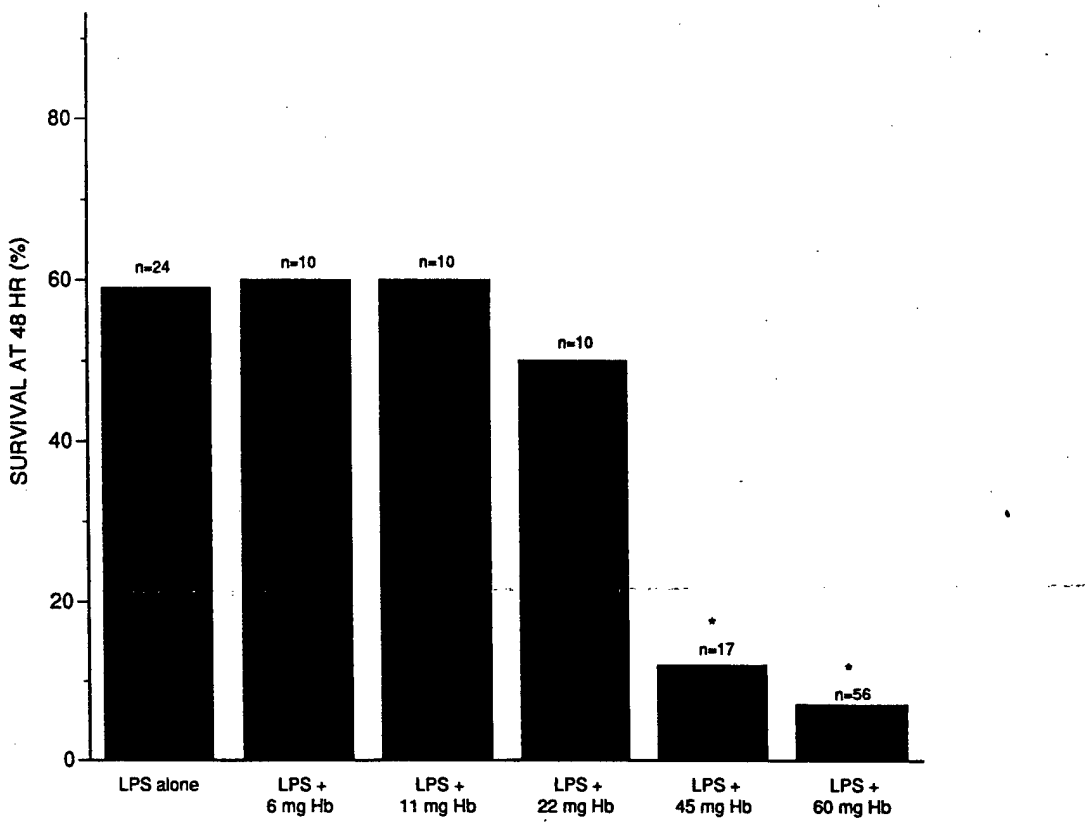

FIG. 2. LPS lethality in mice after the administration of various doses of ααHb. Swiss Webster female mice (28 to 32 g) were injected intraperitoneally with 0.5 mg of LPS (*E. coli* O55:B5 LPS, in sterile, pyrogen-free saline). Eight to 10 h following LPS injection, the mice were infused by tail vein with either 0.6 to 0.8 ml of saline or ααHb in Ringer's acetate (pH 7.4) (doses of Hb ranged from 6 to 60 mg/mouse). Survival was monitored at 48 h. *, $P < 0.01$ versus LPS alone (Fisher's exact P value).

Tissue histology. Tissues were obtained from untreated animals, from animals which had received intraperitoneal LPS 18 h prior to sacrifice, and from animals which had received intraperitoneal LPS 18 h prior to sacrifice and intravenous Hb 8 to 10 h after LPS. Mice were sacrificed by cervical dislocation, and portions of liver, spleen, kidney, lung, heart, adrenal, brain, ovary, uterus, and skeletal muscle were fixed in 10% formalin, sectioned, and stained with hematoxylin and eosin. Tissue sections were examined for evidence of inflammation, hemorrhage, thrombosis, or necrosis.

RESULTS

Effect of Hb on LPS-induced mortality. When 60 mg of ααHb/mouse was infused intravenously 8 to 10 h after intraperitoneal injection of LPS, animals died at a much faster rate than with LPS alone, and overall survival was greatly diminished (Fig. 1). Peak plasma Hb levels achieved by this infusion were approximately 4.0 to 5.5 g/dl. The deleterious effect of Hb was observed at all LPS doses tested (0.1 to 1 mg of LPS/mouse, doses of LPS which alone produced a 20% lethal dose to a 100% lethal dose at 48 h). Seventy-two-hour mortality data were also obtained for the mice which received 0.5 mg of LPS. This dose of LPS produced 72% mortality in the absence of Hb and 96% mortality in the presence of Hb at 72 h. Control experiments demonstrated that infusion of Hb alone (in the absence of LPS) was well tolerated, with no evidence of toxicity (e.g., there was no ruffling of the fur or lethargy and no mortality). Hb enhancement of LPS-induced mortality was dependent on the dose of subsequently infused Hb, as follows: 48-h survival rates with 6 and 11, 22, 45, and 60 mg of Hb/mouse were 60, 50, 12, and 7%, respectively (Fig. 2). Hb infused coincident with LPS also resulted in increased mortality, as did non-cross-linked HbA_0 infused coincident with LPS (Fig. 3). Intraperitoneal injection of preincubated ααHb-LPS mixtures likewise resulted in increased mortality, approximating the mortality observed when Hb was administered intravenously 10 h after LPS. Hb infused intravenously 12 h prior to LPS produced 24-h mortality which was statistically greater than that observed with LPS alone; 48-h survival in the animals which received Hb prior to LPS (36%) was also lower but not statistically different from survival in the animals which received LPS alone (56%).

As for ααHb, infusion of human ββ/ααHb or bovine fumaryl ββHb preparations subsequent to LPS produced greater mortality at both 24 and 48 h than LPS alone (Fig. 3). An additional bovine ββHb produced similar mortality (data not shown). In additional control experiments, infusion of HSA coincident with or subsequent to LPS injection had no statistically significant effect on baseline LPS-induced mortality, in contrast to the synergistic mortality observed with Hb. Finally, to demonstrate that the ability of Hb to enhance LPS mortality was not restricted to the *E. coli* LPS used in the above-described experiments, we also performed experiments with *S. typhosa* LPS. Infusion of Hb 10 h after intraperitoneal injection of *S. typhosa* LPS produced statistically significant enhancement of LPS mortality from 36 to 72 h, similar to the effects of Hb with *E. coli* LPS (data not shown).

We performed a series of experiments to determine whether the enhancement of LPS-induced lethality could be attributed to the heme component of Hb. Preliminary studies indicated that a 0.48-mg dose of hemin chloride (equivalent to the heme

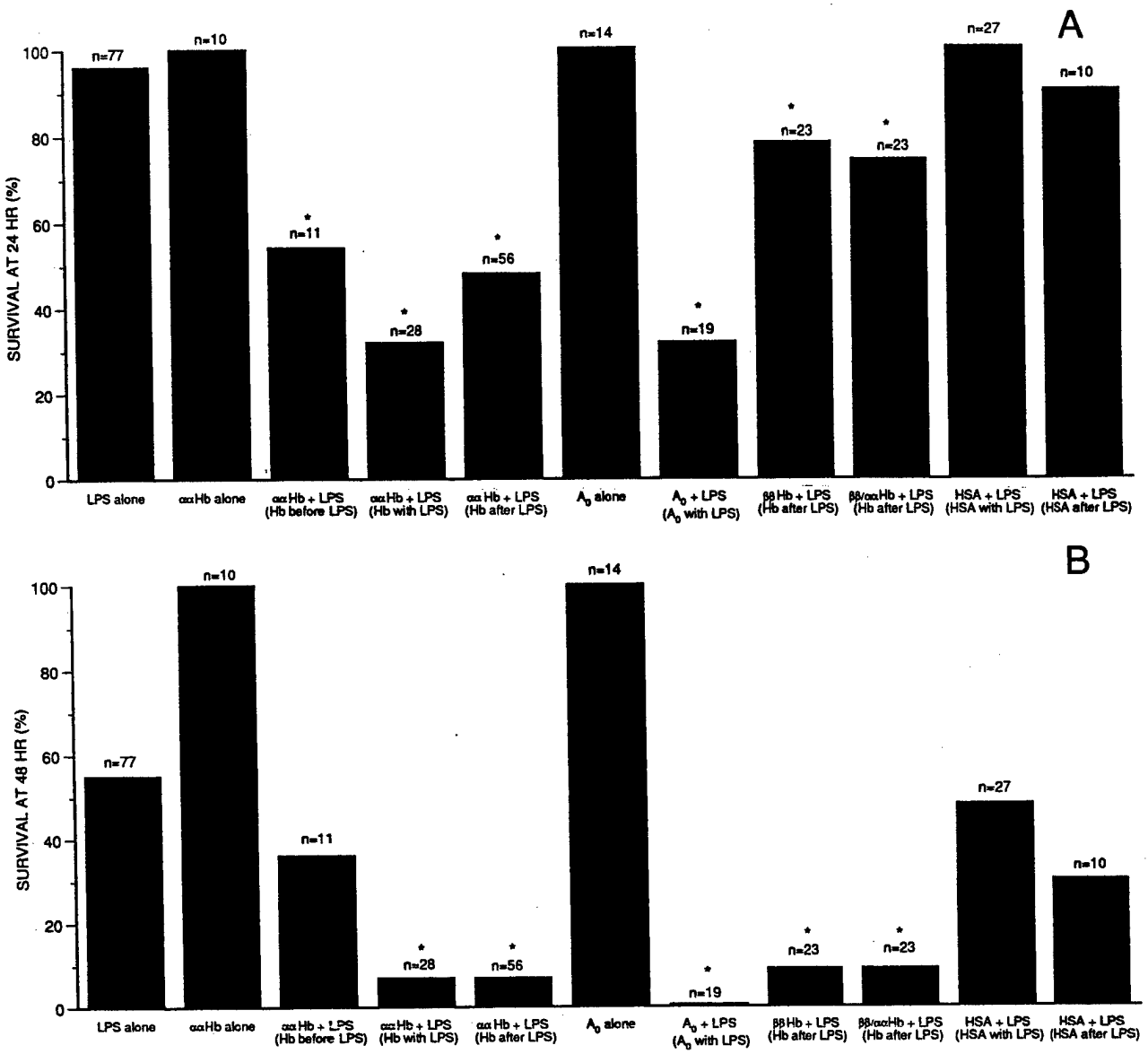

FIG. 3. LPS lethality in mice in the absence and presence of αα Hb, ββ Hb, HbA_0, or HSA. Swiss Webster female mice (28 to 32 g) were injected intraperitoneally with saline or with 0.5 mg of LPS (*E. coli* O55:B5 LPS, in sterile, pyrogen-free saline). Mice also were infused by tail vein with either 0.6 to 0.8 ml of saline, Hb solutions in Ringer's acetate (pH 7.4) (60 mg/mouse), prepared as described in the text, or HSA (60 mg/ml, pH 7.4, in saline with sodium bicarbonate). In various experiments, αα Hb was infused either 12 h before, coincident with, or 8 to 10 h after LPS. Bovine fumaryl ββ Hb and human ββ/αα Hb were infused 8 to 10 h after LPS, and HbA_0 was infused coincident with LPS. HSA was infused either coincident with or 8 to 10 h after LPS. Survival was monitored at 24 h (A) and 48 h (B). *, $P < 0.01$ versus LPS alone (Fisher's exact P value).

content of 12 mg of Hb) could be intravenously administered to mice safely; lethality was observed at ≥1.2 mg of hemin. In four independent experiments, 0.48 mg of hemin did not result in enhanced lethality at either of two LPS doses. Experiments to evaluate the ability of heme-free globin to enhance LPS were not considered feasible because of the known instability of free globin chains.

Endotoxin-resistant mice. LPS was administered to endotoxin-resistant (C3H/HeJ) mice at a dose of 0.5 mg/mouse, corresponding to a 50% lethal dose in normal mice. LPS alone caused no deaths at 48 h in C3H/HeJ mice, and LPS plus Hb caused death in only 14% of these LPS-resistant mice (Fig. 4). For comparison, 48-h mortality in wild-type (Swiss Webster) mice was 94% in mice treated with both LPS and Hb.

Following the demonstration (see above) that Hb enhanced the lethal effects of LPS, we assessed the potential contributions from several possible mechanisms for this result. We considered the possibility that circulating LPS levels were higher in the Hb-treated mice, as well as whether the deleterious systemic response(s) to LPS might be augmented.

Effect of Hb on plasma endotoxin levels. Plasma LPS concentrations became detectably elevated by 30 min after the intraperitoneal administration of LPS and continued to increase for several hours (Fig. 5). Background LPS concentrations in blood obtained from the retro-orbital plexus of untreated animals ranged from undetectable LPS (<0.05 ng/ml) to approximately 2.2 ng/ml. LPS plasma levels at every time point tested were quite variable among mice, but overall, LPS

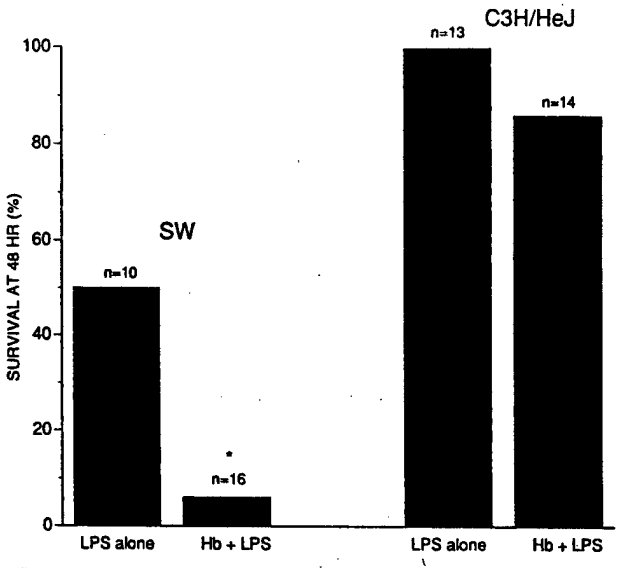

FIG. 4. LPS lethality in Swiss Webster (wild-type) and C3H/HeJ mice in the absence and presence of αα Hb. Swiss Webster (SW) or C3H/HeJ (endotoxin-resistant) female mice (28 to 32 g) were injected intraperitoneally with 0.5 mg of LPS (*E. coli* O55:B5 LPS, in sterile, pyrogen-free saline). Immediately following LPS injection, the mice were infused by tail vein with either 0.6 to 0.8 ml of saline or αα Hb in Ringer's acetate (pH 7.4) (60 mg/mouse). Survival was monitored at 48 h. *, $P < 0.01$ versus LPS alone (Fisher's exact P value).

concentrations peaked at 4 to 10 h and then gradually declined. LPS concentrations measured with the LAL assay were similar to LPS concentrations obtained by isotopic measurement in separate experiments to determine the levels in plasma of ^{3}H-LPS following intraperitoneal administration. For example, the mean LPS plasma concentrations at 4 h after intraperitoneal injection were 20 μg/ml by LAL measurement and 23 μg/ml by isotopic measurement. Absorption into the blood of the tracer ^{3}H-LPS was the same in both the absence and presence of unlabeled LPS (0.5 mg). Following a nadir at 20 h, plasma LPS levels again rose and were typically greater at 34 and 42 h after LPS injection than at 20 h. Intravenous administration of Hb 8 to 10 h after intraperitoneal injection of LPS did not alter the subsequent range of LPS plasma concentrations (Fig. 5). As demonstrated at the 42-h time point, there also was no relationship between plasma LPS concentrations and Hb doses between 6 and 60 mg/mouse. Furthermore, in both the absence and presence of Hb, there was no relationship between LPS plasma concentration, at any of the time periods examined, and subsequent mortality.

Plasma Hb clearance. In mice which received intraperitoneal LPS 8 to 10 h prior to intravenous Hb infusion, there was a dose-dependent effect of LPS on Hb plasma levels (Fig. 6). Persistence of Hb in the plasma was increased (prolonged half-life [$t_{1/2}$]) at 2, 5, and 10 h after infusion by prior injection of 0.5 mg of LPS ($P < 0.02$ versus Hb levels in the absence of LPS). There also was a trend toward slower clearance of Hb in animals which received 0.1 mg of LPS, although the difference from Hb clearance in control animals was statistically significant only at 2.5 h ($P < 0.05$ versus Hb in the absence of LPS). Interestingly, 12 h after Hb infusion, a time at which a substantial quantity of the Hb had been cleared from the circulation, Hb was not detectable in the peritoneal cavity in animals either with or without prior injection of intraperitoneal LPS.

Particulate carbon clearance. The slower clearance of Hb in endotoxemic mice (see above) suggested that there was a depression of reticuloendothelial cell function. Mice were injected with intraperitoneal LPS only, intravenous Hb only, or intraperitoneal LPS followed by intravenous Hb, and reticuloendothelial cell function was assessed by clearance of intravenously injected carbon particles (India ink). Accelerated par-

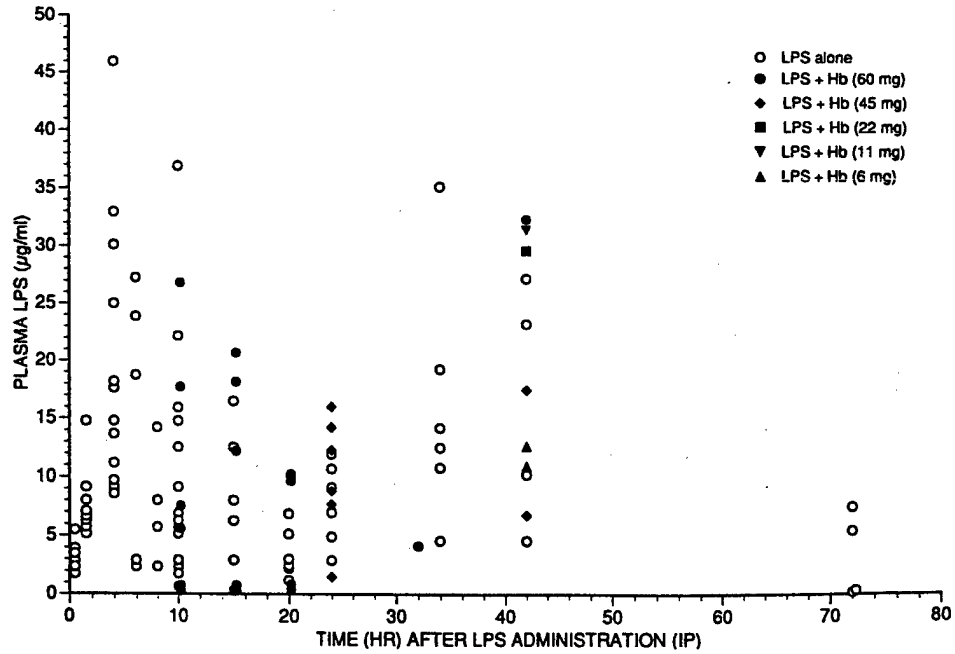

FIG. 5. Plasma LPS concentrations in the absence and presence of αα Hb. Swiss Webster female mice (28 to 32 g) were injected intraperitoneally with 0.5 mg of LPS (*E. coli* O55:B5 LPS, in sterile, pyrogen-free saline). Ten hours after LPS injection, mice were infused by tail vein with either 0.6 to 0.8 ml of saline or αα Hb. The αα Hb dose was 60, 45, 22, 11, or 6 mg/mouse. Blood for LPS levels were serially obtained in EDTA capillary tubes at various time points after LPS injection as described in Materials and Methods. Each data point represents the plasma LPS concentration in a single mouse.

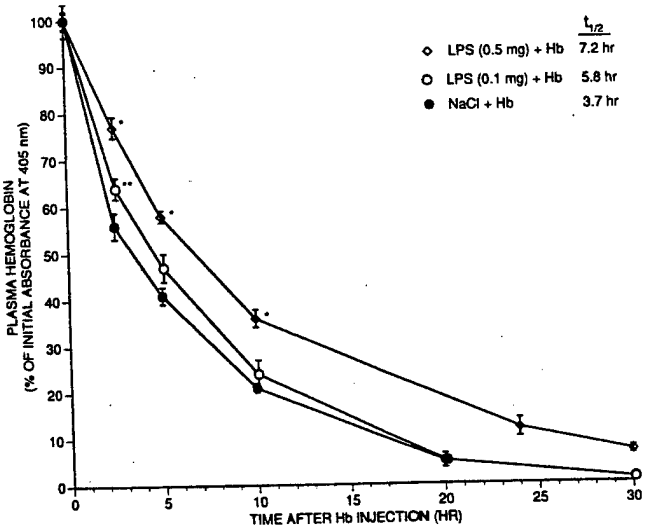

FIG. 6. Hb clearance in mice in the absence or presence of LPS. Swiss Webster female mice (28 to 32 g) were injected intraperitoneally with saline or with 0.1 or 0.5 mg of LPS (*E. coli* O55:B5 LPS, in sterile pyrogen-free saline). Eight to 10 h after injection, mice were infused by tail vein with 0.6 to 0.8 ml of ααHb (60 mg/mouse). Plasma samples were serially obtained with EDTA capillary tubes at various time points after Hb injection. Plasma samples were diluted 1:15 with saline, and absorbances at 405 nm were measured in a plate reader. Initial (t_0) absorbance values at 405 nm, obtained 1 min after Hb infusion, ranged from 1.6 to 2.2, corresponding to plasma Hb concentrations from 4.2 to 5.7 g/dl. Data presented are means ± standard errors for five mice (NaCl), six mice (0.5 mg of LPS), or nine mice (0.1 mg of LPS). *, $P < 0.02$ versus NaCl plus Hb (Mann-Whitney U test); **, $P < 0.05$ versus NaCl plus Hb (Mann-Whitney U test). $P < 0.05$ for 0.1 mg of LPS plus Hb versus 0.5 mg of LPS plus Hb at 2, 5, and 10 h.

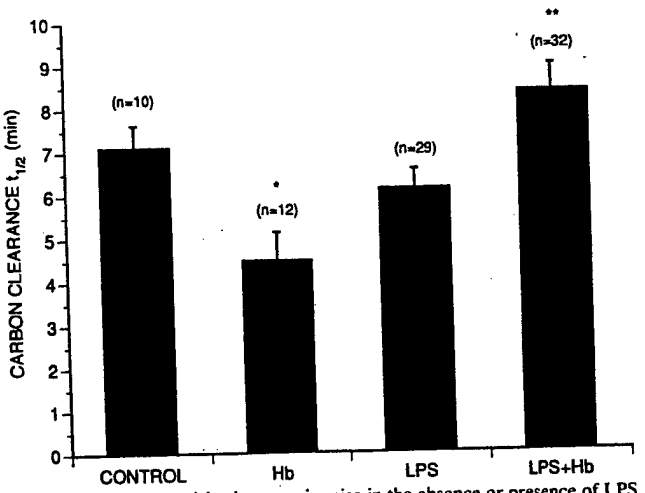

FIG. 7. Carbon particle clearance in mice in the absence or presence of LPS and/or Hb. Swiss Webster female mice (28 to 32 g) were injected intraperitoneally with saline or with 0.5 mg of LPS (*E. coli* O55:B5 LPS, in sterile, pyrogen-free saline). Eight to 10 h after injection, mice were infused by tail vein with 0.6 to 0.8 ml of ααHb (60 mg/mouse) or saline; 10 h later, 0.4 ml of particulate carbon was infused by tail vein. Serial blood samples were obtained with EDTA capillary tubes at various time points (0.5 to 30 min) after carbon injection. Carbon particle clearance rates were calculated for each animal. The results are expressed as the means ± standard errors of the individual $t_{1/2}$ values. *, $P = 0.018$ versus control (Mann-Whitney U test); **, $P = 0.005$ versus LPS alone or Hb alone (Mann-Whitney U test).

ticle clearance (decreased $t_{1/2}$ of clearance) was observed in animals treated with Hb alone compared to untreated animals ($P = 0.018$), indicating stimulation of reticuloendothelial cell function by Hb (Fig. 7). In animals treated with LPS alone, the mean clearance rate was slightly increased but not significantly different from that in untreated animals ($P = 0.21$). In animals treated with both LPS and Hb, the mean clearance rate was significantly decreased (longer $t_{1/2}$) compared to that of the LPS-treated animals ($P = 0.005$), although clearance was not significantly different from that in untreated animals ($P = 0.19$). Therefore, we were able to demonstrate an association between the synergistic lethality produced by LPS and Hb, compared to the lethality of LPS alone. and decreased reticuloendothelial cell function.

Plasma glucose concentrations. Following intraperitoneal LPS injection in subsequently fasted mice, there was a progressive decrease in plasma glucose levels to a broad nadir between 16 and 32 h, followed by a gradual. partial return toward normal at 48 h (Fig. 8). Plasma glucose levels at each time point after 4 h were considerably lower in LPS-treated mice than in fasted control animals ($P < 0.05$). However, at each of the time points tested, there was no difference in plasma glucose concentrations between animals treated with LPS only and those which received LPS plus Hb.

Blood cultures. We considered the possibility that the combination of LPS and Hb had damaged the normal barriers to bacteria entering the bloodstream from the gastrointestinal tract, thus resulting in bacterial sepsis. Blood cultures were obtained from 12 mice which had received both intraperitoneal LPS and intravenous Hb at 26 or 36 h after LPS, times at which mice prominently exhibited the effects of endotoxemia and were beginning to die. Only 1 of 12 blood samples was shown to contain gram-negative bacteria; the remaining cultures remained sterile for 30 days. Therefore, the development of bacterial sepsis was not a significant mechanism of lethality in these mice.

Tissue histology. Histologic evaluation failed to demonstrate any evidence of inflammation, hemorrhage, necrosis, or thrombosis in sections of liver, kidney, lung, brain, ovary, uterus, heart, or skeletal muscle from mice in any treatment group (LPS, Hb, or LPS-Hb). Adrenal glands were also normal, with no evidence of hemorrhage or necrosis. Karyorrhexis, i.e., de-

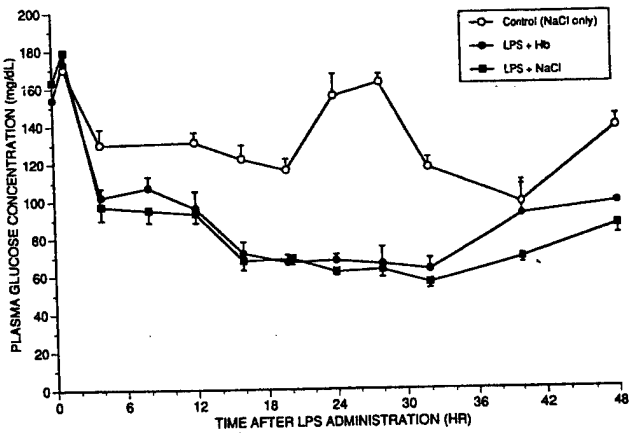

FIG. 8. Plasma glucose concentrations in the absence or presence of ααHb. Swiss Webster female mice (28 to 32 g) were simultaneously injected intraperitoneally with 0.5 mg of LPS (*E. coli* O55:B5 LPS, in sterile, pyrogen-free saline) and intravenously with either 0.6 to 0.8 ml of saline (■; $n = 10$) or ααHb (60 mg/mouse) (●; $n = 13$). All animals were fasted after the beginning of the experiment. Blood for glucose levels was serially obtained in EDTA capillary tubes at various time points after LPS injection. Fasted control mice (○; $n = 8$) received intravenous saline only. Data are expressed as the means ± standard errors of the combination of two independent experiments, each of which demonstrated the differences shown.

struction of nuclei with resultant nuclear fragmentation products, was noted in the splenic white pulp of LPS-treated mice and was even more prominent in LPS-Hb-treated mice; however, there was no acute inflammation (observations not shown).

DISCUSSION

The basis for our studies was the recent recognition that Hb is an LPS-binding protein that, in vitro, enhances many biological activities of LPS (11, 12, 23, 25). A major anticipated use of cell-free Hb as an erythrocyte substitute would be for the emergency treatment of patients with hemorrhage. Associated medical conditions in these patients will likely include severe trauma, gastrointestinal tract ischemia, and bacterial infection. Because of the common occurrence of endotoxemia during these underlying medical conditions, we hypothesized that infusion of Hb into endotoxemic animals might be detrimental. Our studies, which involved large numbers of animals and many independent lethality assays, demonstrated that mortality was significantly greater in mice which received both LPS and Hb than in mice treated with LPS alone. For the majority of our lethality assays, the experimental conditions were purposely chosen such that Hb was given as a bolus infusion into animals with preexisting endotoxemia, after LPS had been absorbed into the bloodstream from an extravascular site (in these studies, the peritoneal cavity) over a prolonged period of time. However, increased toxicity also was observed both in experimental models in which LPS was administered to animals coincident with Hb or after prior Hb infusion and clearance of approximately half of the infused Hb. This is important because endotoxemia in sick patients is characteristically intermittent and prolonged in many clinical scenarios, and the infusion of solutions of Hb into patients with preexisting, coincident, or subsequent endotoxemia is likely in clinical practice. Enhanced lethality was directly related to the infused dose of Hb; synergism was clearly observed with intravenous Hb infusions that generated clinically useful initial plasma Hb concentrations of $\geq$3 g/dl. The synergistic effect of Hb on LPS mortality was a generalized property of all Hb preparations tested, including non-cross-linked HbA_0, α-α-cross-linked preparations, and β-β-cross-linked preparations, but was not observed with the control protein albumin. Therefore, our studies extend the previous observations of White et al. (30) that the intravenous administration of both Hb and LPS was more toxic than administration of LPS alone.

Although Hb alone was well tolerated, it was not certain that the synergistic mortality observed in mice treated with both Hb and LPS was caused solely by an LPS-dependent mechanism. Specifically, it was possible that the process of LPS-induced oxidation of Hb which we recently demonstrated (13) is deleterious in vivo and that Hb-related reactions (e.g., formation of methemoglobin and hemichromes or release of toxic free iron and/or free heme) contribute to mortality. To investigate this possibility, we studied mortality produced by Hb and LPS in a strain of mouse (C3H/HeJ) which is relatively insensitive to LPS, requiring approximately 20 to 50 times more LPS than wild-type mice to obtain a 50% lethal dose (22, 27) and thereby making possible the detection of any Hb-based pathologic effects. There was only a low level of toxicity in C3H/HeJ mice given both LPS and Hb, indicating that any LPS-induced products of Hb oxidation that might have been generated in vivo were not sufficient to account for the high mortality in normal mice that received both LPS and Hb. These results suggested that mortality in the normal endotoxin-sensitive mice was due to LPS-related rather than Hb-related pathophysiological responses.

A series of experiments was performed to evaluate several possible mechanisms for the observed synergistic toxicity of Hb and LPS. The indication that mortality was based on LPS-related pathophysiological responses (rather than Hb-related oxidative processes) and the LPS-binding property of Hb suggested that alteration by Hb of LPS absorption and/or clearance, leading to higher LPS blood levels, might provide a possible mechanism for Hb enhancement of LPS mortality. Therefore, we evaluated whether Hb infusion 8 to 10 h after LPS administration (the standard conditions used to obtain the data presented in Fig. 1 and 2) resulted in LPS plasma levels that were higher than in the absence of Hb. However, at all time points examined, the ranges of plasma LPS concentrations were not different between mice which received LPS only and those which received LPS plus Hb. Therefore, there was no evidence that Hb infusion influenced either the continuing absorption of LPS or its clearance, and accordingly, enhanced mortality could not be correlated with plasma LPS levels. Interestingly, in both groups there was a nadir in the plasma LPS concentrations at 20 h after intraperitoneal injection, followed by increased LPS levels in several mice at 34 and 42 h. Many of these animals appeared extremely toxic at these late time points. We speculate that the high LPS levels that developed later than 24 h after administration of LPS might result from a damaged or inflamed peritoneum that had become more permeable to the injected endotoxin, thus permitting increased entry of LPS into the circulation. Perhaps continued absorption of the intraperitoneally injected LPS in association with a decreased intravascular clearance rate accounts for the elevated levels. Alternatively, at the later time points, the high LPS levels might reflect translocation of gut-derived LPS into the circulation. Interestingly, translocation of live bacteria from the gastrointestinal tract into the bloodstream, a process which could potentially result in lethal bacterial sepsis, was a rare event.

The $t_{1/2}$ of a bolus infusion of Hb was approximately doubled in mice pretreated with LPS (0.5 mg). This prolonged persistence of Hb in the presence of LPS suggested that intravascular clearance mechanisms were abnormal in these mice. Since the cross-linked preparation of Hb used in these experiments is primarily cleared by tissue macrophages (with negligible formation of dimers which could lead to renal clearance), our results suggested that the combination of LPS and Hb might have resulted in depression of normal reticuloendothelial system clearance mechanisms. Attenuated killing of microorganisms by Kupffer cells has previously been proposed as a mechanism for enhanced mortality from gram-negative bacteria in the presence of Hb (14, 15). Furthermore, sensitivity of animals to LPS (e.g., increased pyrogenic response) has previously been associated with depressed reticuloendothelial cell function (e.g., after Thorotrast blockade) (1), although other studies have found an association between sensitivity to LPS (e.g., enhanced mortality) and stimulated reticuloendothelial system function (e.g., after glucan [6] or glycerol trioleate [26] administration). We reasoned that if initial uptake of LPS and Hb saturated these cells sufficiently to subsequently decrease their particle clearance capacity, the cells might not function normally, and depressed reticuloendothelial cell function then could contribute to the observed enhancement by Hb of LPS-induced mortality. To further examine this possibility, we studied the influence of LPS alone, Hb alone, and LPS plus Hb on reticuloendothelial cell function as measured by clearance of particulate carbon. Hb alone stimulated carbon clearance, a result in agreement with the previously reported effect of po-

lymerized Hb on reticuloendothelial cell function (20). Intraperitoneal LPS alone had no demonstrable effect in our study of reticuloendothelial cell function, although high doses of LPS administered intravenously are known to depress the reticuloendothelial cell system (2). However, the combination of Hb and LPS produced a depression in reticuloendothelial cell function compared to the effect of LPS alone, suggesting that reticuloendothelial system dysfunction may contribute to the observed synergistic mortality that followed administration of Hb plus LPS. Nevertheless, it remains uncertain whether a direct toxic effect of Hb and LPS on the reticuloendothelial cells, or rather a decrease in hepatic and splenic perfusion, resulted in the depressed reticuloendothelial cell function associated with the combined administration of LPS and Hb.

Since we had shown previously that cellular responses to LPS, in vitro, are enhanced by LPS (11, 23, 25), we thought it possible that Hb might result in increased mortality by augmenting the hypoglycemia associated with LPS. Animals were fasted during this experiment to ensure that the sicker LPS-Hb group of mice did not have caloric deprivation different from that of the LPS-only group of mice. The two groups demonstrated similar levels of hypoglycemia in excess of that found in fasted control mice, with no effect of Hb. Interestingly, both groups also demonstrated partial recovery of glucose levels after 30 h, and therefore it is not certain the extent to which hypoglycemia contributed to mortality in either group of mice.

Finally, since both Hb and LPS are cleared primarily via Kupffer cells and splenic macrophages, and these cells are major sources of pathologic inflammatory mediators, it is possible that Hb and LPS cleared by an altered reticuloendothelial system are processed slowly, leading to their prolonged interaction with reticuloendothelial cells and resultant enhancement of deleterious host cytokine responses. We have begun to study the influence of Hb on the production of tumor necrosis factor (TNF) as a likely candidate for such a cytokine-mediated mechanism. TNF is produced early in the response to LPS and is believed to be a major mediator of the diverse pathophysiologic responses to LPS (3, 21, 28). In preliminary experiments, we have observed higher plasma TNF levels in mice which received both LPS and Hb than in mice treated with LPS only. These preliminary findings need to be confirmed and expanded, and additional investigations will be required to assess whether differences in production of other proinflammatory and/or anti-inflammatory cytokines provide additional mechanisms for the synergistic toxicity of Hb and LPS.

In conclusion, LPS-related mortality is greatly increased by a variety of forms of cell-free Hb. However, the mechanism for this effect remains unclear. This finding is of clinical interest because of the likely presence of endotoxemia in many patients who would be transfused with solutions of Hb. It is also possible that an interaction between Hb and LPS contributes to the Hb-enhanced mortality associated with gram-negative bacterial sepsis which has recently been demonstrated with both native (unmodified) and polymerized pyridoxalated Hb (8).

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Research

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Effects of iron on bacterial endotoxin

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Summary Bacterial endotoxin, a major modulator of morbidity and mortality during bacterial sepsis, has affinity for cations. Binding of certain cations to LPS can cause alterations of LPS size and ultrastructure, and can affect LPS biological potency. Although the addition of iron to LPS has been shown previously to result in decreased LPS-mediated lethality in mice, it is not certain whether this represents a direct effect of iron on LPS biological activity. To examine this possibility, we measured binding of ferrous or ferric iron to *Escherichia coli* LPS in vitro. 1.5–2 moles of iron (regardless of oxidation state) was shown to bind per mole LPS. Binding of iron to LPS produced a dose-dependent decrease in two measures of LPS biological activity, activation of *Limulus* amebocyte lysate (which decreased to 10% of control when LPS was saturated with Fe) and stimulation of endothelial cell procoagulant activity (which decreased to 1–10% of control when LPS was saturated with Fe).

INTRODUCTION

Bacterial endotoxin (lipopolysaccharide, LPS), the toxic component of the Gram-negative bacterial cell wall responsible for many of the pathophysiological effects of sepsis, contains a variety of tightly bound cations, in particular sodium, calcium, zinc, magnesium, and iron, and basic amines such as spermine.[1,2] The cation content of LPS can have a substantial influence on the macromolecular aggregation state and ultrastructural shape of LPS,[1,3–5] which in turn can affect LPS biological activities.[3,6] Of these bound cations, iron is of particular interest because of its important role in bacterial growth and the host's attempt to control infection via production of hypoferremia.[7,8] The observation that iron metabolism is often deranged (low serum iron and increased reticuloendothelial cell iron) at the time that endotoxemia is present during sepsis has led others to examine the effect of iron on LPS-induced lethality. Addition of iron to LPS has been associated with a decrease in LPS-induced lethality in mice,[9,10] although the relative importance of ferrous and ferric iron in this process is unclear. It has been suggested that such protection from LPS may involve, in part, iron-induced alterations in reticuloendothelial cell system function,[9] although changes in LPS potency also have been postulated.[2]

Recent studies in our laboratory have examined toxicities associated with the use of cell-free hemoglobin, a red blood cell substitute, in septic recipients. We have demonstrated that cell-free hemoglobin (Hb) dramatically enhances LPS-mediated mortality in mice,[11] possibly via binding to and subsequently increasing the potency of LPS, an effect we have extensively demonstrated in vitro.[12–14] An interesting consequence of formation of complexes between LPS and Hb is an acceleration in Hb autooxidation, with changes in intensity of the Soret peak of Hb that are consistent with partial loss of heme and iron from Hb.[15] Given the known ability of LPS to bind iron, it seemed likely that iron lost from Hb during oxidation might become complexed with LPS. The following experiments were undertaken to measure the capacity of LPS to bind iron, and to determine whether LPS subsequently exhibited altered biological activity.

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MATERIALS AND METHODS

Reagents

Ferrous sulfate, ferric chloride, and hydroxylamine HCl were obtained from Sigma (St Louis, MO, USA). Smooth *E. coli* lipopolysaccharide 055:B5, prepared by trichloroacetic acid extraction, was obtained from Difco Laboratories (Detroit, MI, USA). Rough *Salmonella minnesota* R60 (Ra) lipopolysaccharide, deep rough *S. minnesota* R595 (Re) lipopolysaccharide, and *S. minnesota* lipid A, prepared by phenol-chloroform-petroleum ether extraction, were obtained from List Biological Laboratories, Inc. (Campbell, CA, USA). Lipopolysaccharides had low levels of contamination by protein (all < 1.5%) or nucleic acid (all < 1.5%). Sterile, endotoxin-free water and 0.9% NaCl were purchased from Travenol Laboratories (Deerfield, IL, USA). Chromogenic substrate S-2423 was obtained from AB Kabi Vitrum (Molndal, Sweden). Human umbilical vein endothelial cells and cell culture medium were obtained from Clonetics Corp. (San Diego, CA, USA). Sterile tissue culture plasticware was obtained from Becton Dickinson (Mountain View, CA, USA).

Production of LPS-iron complexes

LPS (0.25 mg/ml) was prepared in water by vortexing for 2 min followed by brief sonication (30 s). Ferrous sulfate in 1.5% hydroxylamine (pH 4.5), and ferric chloride in water (pH 2.4), were each prepared at 100 μg/ml and diluted as needed. Equal volumes of LPS and iron (1.25 ml each) were mixed and incubated overnight at room temperature (20°C). Control mixtures without iron contained LPS and hydroxylamine buffer. In selected experiments, a range of incubation temperatures (4°C, 20°C, and 37°C) or incubation times (10 min, 1 h, 18 h) were studied. LPS-iron complexes were separated from unbound iron by chromatography on a PD-10 desalting column (Pharmacia Biotech Inc., Piscataway, NJ, USA). Iron bound to LPS was measured with a quantitative iron assay based on its reaction with ferrozine, according to the manufacturer's instructions (Sigma). Briefly, 0.4 ml sample, 0.4 ml hydroxylamine buffer (1.5% in acetate, pH 4.5), and 0.05 ml ferrozine (0.85% in hydroxylamine buffer) were incubated at 37°C for 30 min to produce ferrozine-iron complexes, and absorbances then were measured at 560 nm. The iron standard was in hydroxylamine buffer (Sigma). Iron intrinsic to the preparations of LPS was <0.03 μg/mg LPS.

Limulus amebocyte lysate (LAL) assay

Amebocyte lysates were prepared from *Limulus polyphemus* (the North American horseshoe crab) by lysis of washed amebocytes in distilled water, as described previously.[16] Activation of LAL by LPS and by LPS-iron complexes (produced as described above) was quantified in 6 replicates with a chromogenic LAL test, as described previously.[12] LAL activation was monitored by a time-dependent increase in absorbance at 405 nm, a measure of proteolysis of the chromogenic substrate.

Endothelial cell tissue factor assay

Human endothelial cells (HUVEC) were plated in 96-well microtiter plates (Nunclon, Applied Scientific, South San Francisco, CA, USA) in 0.1 ml media containing 4% serum, as described previously.[14] Cells were utilized at less than 6 passages. Confluent endothelial cell monolayers were incubated with LPS or LPS-iron complexes (produced as described above) for 4 h at 37°C in 10% CO_2, and tissue factor (TF) procoagulant activity in the disrupted monolayers measured as described previously using a plasma recalcification assay.[14] TF activity was calculated from the turbidity (fibrin clot) generated in the lysed HUVEC-plasma mixture following the addition of calcium, based on a standard curve established with dilutions of rabbit brain thromboplastin (Baxter Corporation, Miami, FL, USA). The turbidity generated at 8 min by 1:100 diluted thromboplastin was defined as 1 TF arbitrary unit. Mean TF activity in samples was based on 6–8 replicate wells.

RESULTS

When ferrous sulfate solutions and LPS were incubated and the LPS-iron complexes isolated by chromatography, a concentration-dependent binding of Fe^{2+} to LPS was observed, with a maximum of approximately 7 μg Fe^{2+} bound/mg LPS (Fig. 1). Scatchard analysis of the binding curve demonstrated a k_D = 113 μM and a maximum stoichiometry of 2 moles Fe^{2+} per mole LPS (based on a monomer molecular weight of 12 000 for this *E. coli* LPS). Similar binding curves were obtained when the incubations of ferrous iron and LPS were performed unbuffered (data not shown). Binding curves also were unaffected by the temperature of incubation (4°C, 20°C or 37°C) or by the time of incubation (10 min, 1 h, 18 h) (data not shown). Concentration-dependent binding of ferric chloride to LPS similarly was observed (Fig. 2), with a maximum of approximately 5 μg Fe^{3+} bound/mg LPS, representing a stoichiometry of approximately 1.5 moles Fe^{3+} per mole LPS, and a k_D = 130 μM.

To obtain information regarding the binding site(s) on LPS for iron, binding experiments were performed with smooth LPS, Ra LPS composed of lipid A plus the complete inner and outer core regions, Re LPS composed of lipid A plus KDO, and lipid A alone (Fig. 3). No

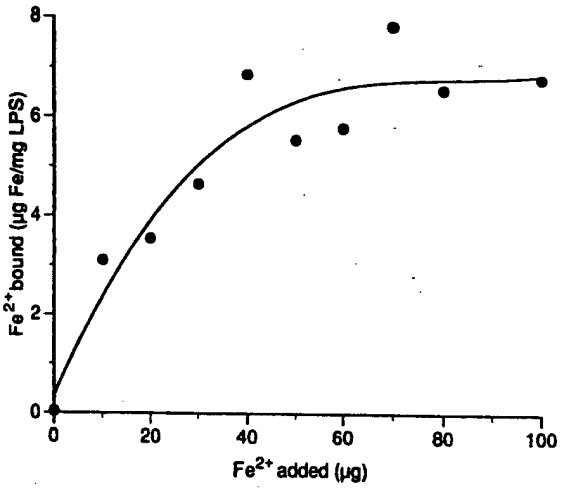

Fig. 1 Binding of Fe^{2+} to LPS. Ferrous sulfate in hydroxylamine (pH 4.5) was incubated with *E. coli* LPS, and bound iron separated from unbound iron by chromatography on a PD-10 column. Fe bound to LPS was determined in triplicate by a quantitative iron assay. The iron content of LPS alone, 0.03 µg Fe/mg LPS, has been subtracted from each of the data points.

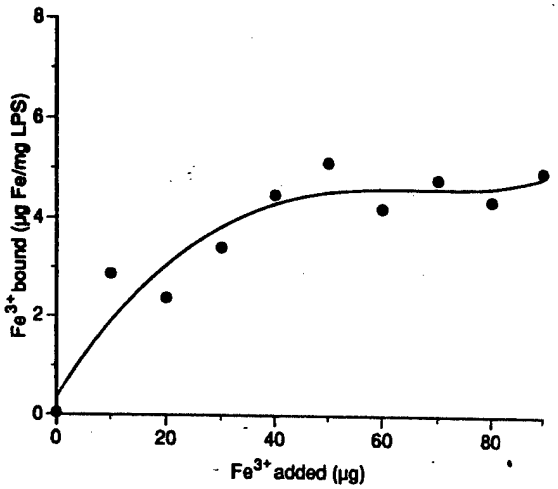

Fig. 2 Binding of Fe^{3+} to LPS. Ferric chloride in water was incubated with *E. coli* LPS, and bound iron separated from unbound iron by chromatography on a PD-10 column. Fe bound to LPS was determined in triplicate by a quantitative iron assay. The iron content of LPS alone, 0.03 µg Fe/mg LPS, has been subtracted from each of the data points.

differences were apparent in the binding of ferrous iron to these various LPSs, suggesting that sites on lipid A, possibly the phosphoryl groups, were involved in iron binding. There was no evidence that iron had bound to the core or O-chain regions of LPS.

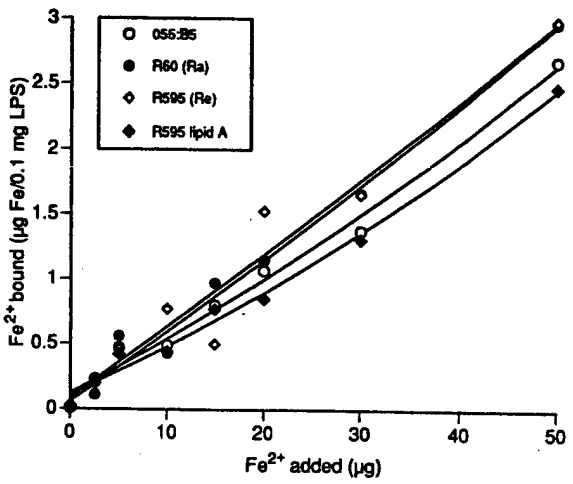

Fig. 3 Binding of Fe^{3+} to a variety of chemically distinct LPSs. Ferrous sulfate in hydroxylamine (pH 4.5) was incubated with smooth *E. coli* 055 B:5 LPS, rough *S. minnesota* R60 (Ra) LPS, deep rough *S. minnesota* R595 (Re) LPS, and *S. minnesota* lipid A, and bound iron separated from unbound iron by chromatography on a PD-10 column. Fe bound to LPS was determined in triplicate by a quantitative iron assay. The iron content of LPS alone, 0.02–0.05 µg Fe/mg LPS, has been subtracted from each of the data points.

A series of LPS-iron complexes, formed with different concentrations of Fe^{2+} and then isolated by chromatography, were diluted in water to ~2 µg/ml LPS, and then compared to LPS alone (also isolated by chromatography) for potency in activating LAL. Increasing concentrations of iron in the initial incubation mixtures with LPS (*E. coli* 055:B5) resulted in a concentration-dependent decrease in LPS biological activity (Fig. 4). This conclusion was based on the observed increase in lag time before appearance of significant absorbance at 405 nm, and a decrease in the slope of the absorbance curves in the LAL assay for LPS as the initial concentration of Fe^{2+} was increased. The chromogenic absorbance curve obtained with LPS-iron complexes isolated from incubations that had contained the highest concentration of iron (filled squares), and which demonstrated the greatest extent of LPS inhibition, indicated LPS equivalent to approximately only 10% of the biological activity of the same concentration of LPS in the absence of iron (open circles). Addition of ferric (Fe^{3+}) iron produced a concentration-dependent decrease in LPS biological activity similar to that seen with ferrous iron (data not shown).

Isolated LPS-iron complexes, containing Fe^{2+} as shown in Figure 1, also were examined for induction of endothelial cell procoagulant activity (tissue factor, TF) by LPS. LPS alone (*E. coli* 055:B5) and LPS-iron complexes were diluted in water to 50 µg/ml LPS, and added to cultured endothelial cells; tissue factor induced by LPS then was measured with the plasma recalcification assay. Iron caused a concentration-dependent inhibition of LPS-

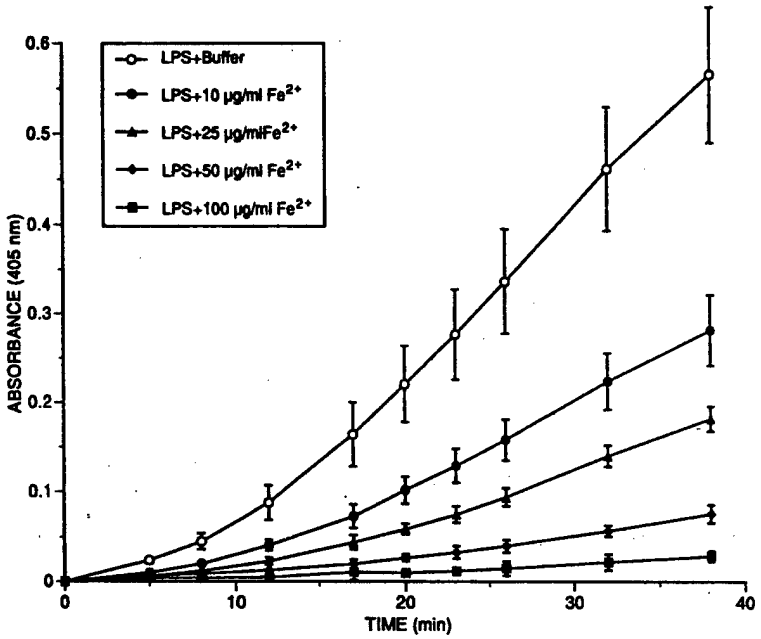

Fig. 4 Effect of Fe^{2+} on LPS biological activity in the LAL assay. Ferrous sulfate (10–100 μg/ml, final concentration) was incubated with *E. coli* LPS, and bound iron separated from unbound iron by chromatography on a PD-10 column. LPS alone and Fe-LPS complexes were assayed for LPS biological activity in a chromogenic LAL assay, in which LAL activation by LPS produces a time-dependent increase in absorbance at 405 nm. Means ± 1 SD of six replicate measurements are presented. Representative of four independent experiments.

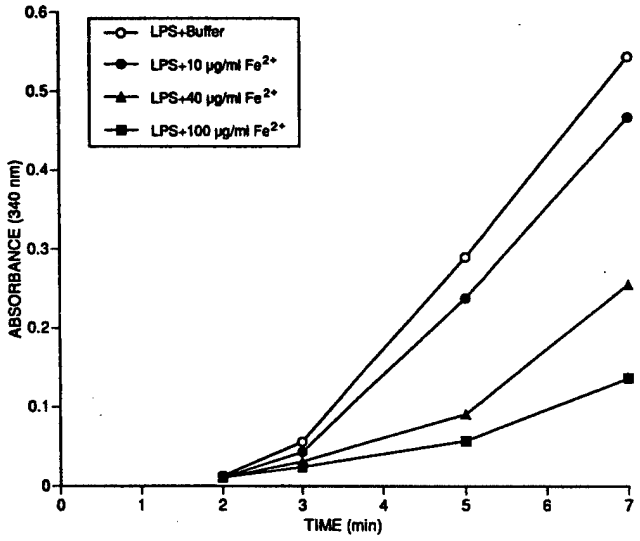

Fig. 5 Effect of Fe^{2+} on LPS biological activity in an endothelial cell procoagulant assay. Ferrous sulfate (10–100 μg/ml, final concentration) was incubated with *E. coli* LPS, and bound iron separated from unbound iron by chromatography on a PD-10 column. LPS alone and Fe-LPS complexes were assayed for LPS biological activity by measurement of LPS-induced tissue factor (procoagulant activity) from cultured human umbilical vein endothelial cells. Tissue factor was assayed in a plasma recalcification assay, in which tissue factor-induced clotting of plasma produces a time-dependent increase in absorbance at 340 nm (turbidity). Means of 6–8 replicate measurements are presented. Representative of three independent experiments.

induced procoagulant activity, as measured by the rate of plasma clot formation (absorbance at 340 nm; Fig. 5). Maximal inhibition by iron (which resulted from complexes obtained from incubations of LPS with 100 μg/ml iron) produced a 4-fold decrease in tissue factor activity when expressed as tissue factor units, based on a thromboplastin standard curve (Fig. 6). In previous studies, a 4-fold decrease in LPS-induced tissue factor activity was produced by a 10–100-fold decrease in LPS concentration.[14] Therefore, LPS biological activity in both the LAL and tissue factor assays was similarly decreased by iron.

DISCUSSION

LPS, extracted from Gram-negative bacterial cell walls, contains numerous cations, prominently including Na, Mg, Ca, and Zn.[1,2] Concentrations of these cations in specific preparations of LPS vary greatly, and range from approximately 0.1–30 μg cation/mg LPS.[1,2] In contrast, iron is present in lower concentrations in LPS (0.05–0.1 μg/mg LPS).[2] In the present study, we have demonstrated that substantial additional quantities of iron (5–7 μg/mg LPS), in either the divalent or trivalent state, are capable of binding to LPS. Under conditions of excess Fe, the resultant molar stoichiometries of Fe and LPS in the complexes were approximately 1.5:1 to 2:1, respectively.

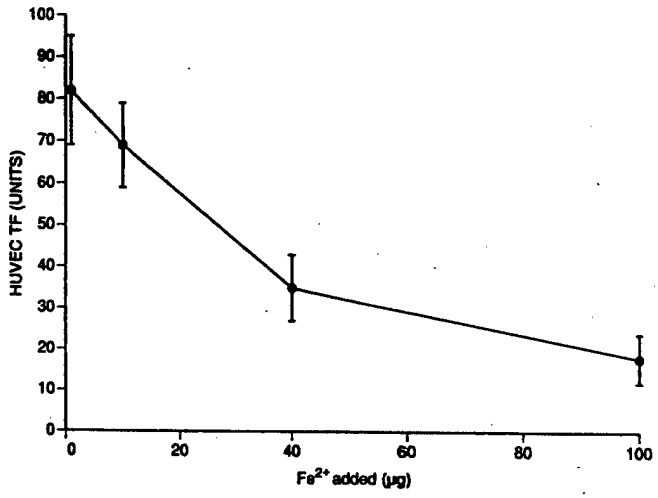

Fig. 6 Effect of Fe^{2+} on LPS biological activity in an endothelial cell (HUVEC) procoagulant assay. Tissue factor (TF) production by LPS and Fe-LPS complexes, measured by the curves for plasma coagulation presented in Figure 4, has been quantified and expressed as TF units using a thromboplastin standard curve. Means ± 1 SD are shown.

The measured affinity of binding for Fe^{2+} and LPS (k_D = 113 μM) was lower than that reported previously for the high affinity LPS binding site for Ca^{2+} ($k_D = 10^{-8}$ M) but greater than the low affinity Ca^{2+} site ($k_D = 10^{-3}$ M).[4]

In the present study, we demonstrated with two independent assays of LPS biological activity (LAL activation and endothelial cell tissue factor production) that the binding of Fe to LPS reduced LPS potency. Therefore, it is possible that the decrease in LPS-induced lethality in mice that has been observed with LPS preincubated with Fe[9,10] may have resulted from the production of low potency LPS. Alternatively, based on the binding stoichiometries that we have determined, the incubation mixtures used for the in vivo studies[9,10] would likely have contained large amounts of unbound Fe, and thus a beneficial effect of the infusion of free Fe (e.g. a protective effect of Fe-induced stimulation of the reticuloendothelial cell system, as suggested previously[9]), cannot be ruled out. However, in other investigations, the separate injections of Fe and LPS, without preincubation, did not result in less lethality than LPS alone,[10] suggesting that direct contact between the two agents is required to produce LPS detoxification. Although the mechanism of LPS detoxification by iron is not known, other studies in our laboratory have ruled out chemical degradation of LPS. Specifically, in the presence of iron and hydroxylamine, there is no demonstrable dephosphorylation of lipid A (as assessed by thin layer chromatography), nor is there detectable deacylation of lipid A (as assessed by quantification of released free fatty acid).

The current experiments were undertaken to investigate aspects of the interaction of LPS with cell-free hemoglobin, a process that has been studied extensively in our laboratory. Specifically, we have demonstrated that the binding of LPS to hemoglobin induces hemoglobin oxidation[15] and an increase in LPS biological activity both in vitro[12–14] and in vivo.[11] Hemoglobin oxidation can result in heme loss and the generation of toxic free iron. Accordingly, we considered the possibility that if LPS-induced hemoglobin oxidation resulted in iron loss, the free iron might bind to LPS and consequently affect LPS biological activity. Effects of cation content of LPS on LPS three-dimensional structure and endotoxicity are well known (for reviews, see Galanos & Lüderitz[3] and Rietschel et al[6]) and, therefore, it was reasonable to assume that Fe transferred from hemoglobin to LPS during hemoglobin oxidation might influence LPS potency. However, the detoxifying effects of free Fe on LPS activity that we have demonstrated in the present study indicate that binding of Fe to LPS does not explain the enhanced in vitro and in vivo biological activities of LPS-hemoglobin complexes compared to LPS alone. Therefore, if cell-free hemoglobin infused into patients as a red blood cell substitute interacts with LPS during concomitant sepsis, we do not believe that the potential transfer of Fe from hemoglobin to LPS is likely to contribute to the pathological consequences of the interaction.

Bacteria are known to utilize mammalian host sources of iron for their growth and pathogenicity, and responses that diminish iron availability to bacteria are important for host control of infectious diseases.[7,8] Bacterial siderophores, compounds with very high affinity for host iron, are able to acquire sufficient iron to ensure microbial viability.[8] However, it is unknown whether LPS may act to bind additional iron during infection. The present studies suggest that such a process would decrease LPS potency and consequently decrease the pathophysiological responses to endotoxemia. Therefore, it is interesting to speculate that hypoferremia, which typically accompanies sepsis and is generally believed to aid in the host defense against bacterial infection, might paradoxically limit a potential mechanism to detoxify LPS during sepsis.

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Interactions Between Hemoglobin and Bacterial Endotoxin In Vitro and In Vivo

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I. INTRODUCTION

Toxicities of hemoglobin (Hb) solutions, which have been demonstrated in numerous animal resuscitation models, prominently include fever, hypertension, thrombocytopenia, activation of the complement and coagulation cascades, disseminated intravascular coagulation with parenchymal organ damage, reduced tolerance to sepsis, susceptibility to bacterial infections, reticuloendothelial cell blockade, and lethal toxicity (1–10). In addition, recent clinical trials of cross-linked Hb have been associated with production of hypertension and gastrointestinal dysmotility.

It was recently demonstrated that injection of nonlethal doses of gram-negative bacteria into mice produced 50 and 100% mortality when the animals had been preinfused with either native or cross-linked preparations of cell-free Hb, respectively (11). Mortality was lessened when sepsis was not produced until 3 hr after administration of either Hb. However, these results were not confirmed in apparently similar models that were performed with either mice (12) or rats (13). In vitro, Hb has been shown to stimulate production of tissue factor by mononuclear cells (14), cause endothelial cell injury (15) and activate complement (14). These in vivo and in vitro effects are characteristic of bacterial endotoxins (lipopolysaccharide, LPS). Investigations of the possibility that

LPS may contribute to the observed side effects of Hb infusions have been a major focus of our laboratory during the past several years, and a significant role for LPS in Hb toxicity has been suggested by our studies.

One of the most critical aspects of LPS toxicity is the high in vivo potency of LPS, even at very low concentrations (pg/mL). During the preparation of Hb-based resuscitation fluids, LPS is a potentially ubiquitous contaminant; even low levels of LPS contamination become a major clinical concern when large volumes of resuscitation solutions are required for infusion. In addition, physiologically significant levels of LPS are present in the circulating blood in a variety of clinical conditions, including sepsis, hepatic injury, hypotension, and damage to the gastrointestinal tract. Because many clinical circumstances for which Hb-based resuscitation fluids would be administered are likely to be associated with shock and hypoxia (pathological states that lead to deterioration of mucosal barriers and hepatic function), significant concentrations of endotoxin would be expected to be potentially present in the circulation of many patients. Since there is increasing evidence that cell-free Hb and LPS synergistically produce toxicities, the infusion of Hb-based resuscitation fluids may potentiate the toxicity of preexisting endotoxemia (or of endotoxemia that subsequently occurs when Hb remains present in the plasma), thus compounding the problem of the high intrinsic biological potency of LPS. The coinfusion of LPS and Hb into rabbits activated blood coagulation and produced a marked increase in mortality (50–100%) compared to the toxicity of LPS or cell-free Hb alone (only 0–10%) (16). We have shown that LPS clearance in vivo is retarded in the presence of hemoglobinemia. The biological effects of LPS in vitro, such as activation of coagulation mechanisms (both the direct activation of coagulation cascades and the production of monocyte and endothelial cell-derived procoagulant activity), can be enhanced up to 100-fold by cell-free Hb. Furthermore, rates of Hb oxidation to methemoglobin and hemichromes are dramatically increased in the presence of LPS. Thus, the binding of cell-free Hb to LPS produces complexes that result both in enhancement of the biological activities of LPS and degradation of Hb.

Our experience in the field of blood substitute research has been with Hb solutions, including both native human HbA_0 and cross-linked Hb [human Hb, $\alpha\alpha$ cross-linked using bis(3,5-dibromosalicyl) fumarate (DBBF); and bovine Hb, fumaryl $\beta\beta$ cross-linked]. Investigations in our laboratory during the past several years have led to an understanding of the complex contributions of LPS to the observed toxicities of Hb solutions. Initial experiments suggested the possibility that Hb was a previously unrecognized LPS-binding protein. Subsequently, detailed experiments documented the formation of Hb-LPS complexes, characterized the complexes, and identified consequences of the LPS-Hb interaction that might contribute to toxicity.

II. DEMONSTRATION THAT Hb IS AN LPS-BINDING PROTEIN

An extensive series of experimental approaches have been utilized to document that mixtures of LPS and Hb produce stable complexes (17). In all experiments, equivalent results were obtained using either purified native, unmodified human HbA_0 or cross-linked Hb prepared as a potential red blood cell (RBC) substitute. Direct evidence of saturable binding of LPS to immobilized Hb was obtained (Fig. 1). The calculated K_d [4.7×10^{-4} g/L (3.1×10^{-8} M, assuming a monomer molecular mass of 1.5×10^4 for *E. coli* LPS) based on the microtiter plate binding assay and 6.3×10^{-4} g/L based on a sucrose centrifugation assay] indicated that the interaction between Hb and LPS is of moderate affinity. Complex formation also was demonstrated by affinity labeling of Hb with a photoactivatable form of LPS (Fig. 2). Using density gradient centrifugation, comigration of LPS with Hb was shown, and it was demonstrated that the sedimentation velocity of LPS was decreased in the presence of Hb preparations (Fig. 3). This indicated that there had been disaggregation of LPS and

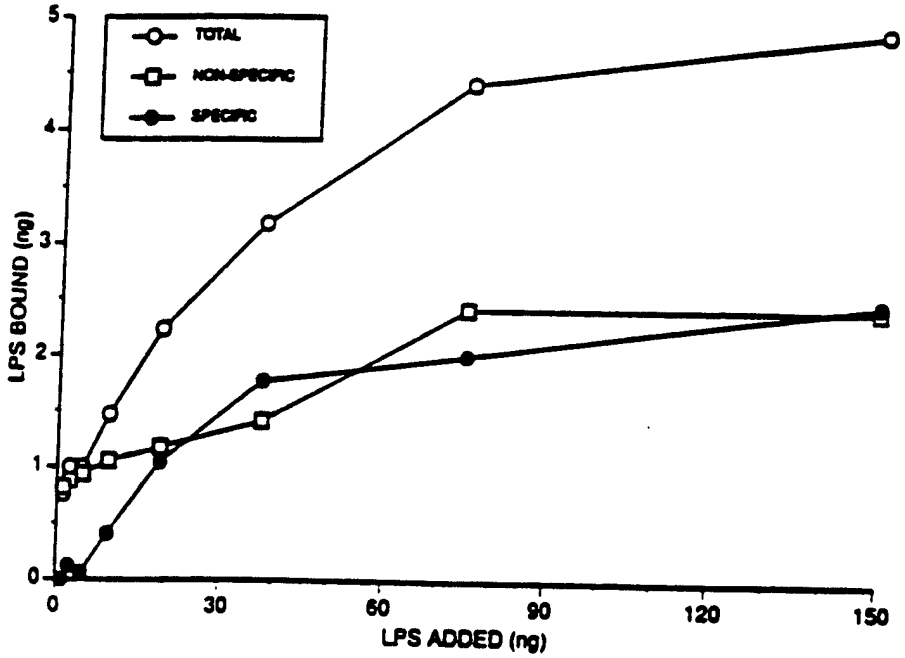


Figure 1 Binding of LPS to immobilized Hb. $\alpha\alpha$Hb (1 μg per well) was immobilized in microtiter plate wells, and ^{125}I-LPS was added. Bound LPS was determined by gamma counting, and specific binding was calculated by subtracting bound ^{125}I-LPS in wells without Hb. (From Ref. 17.)

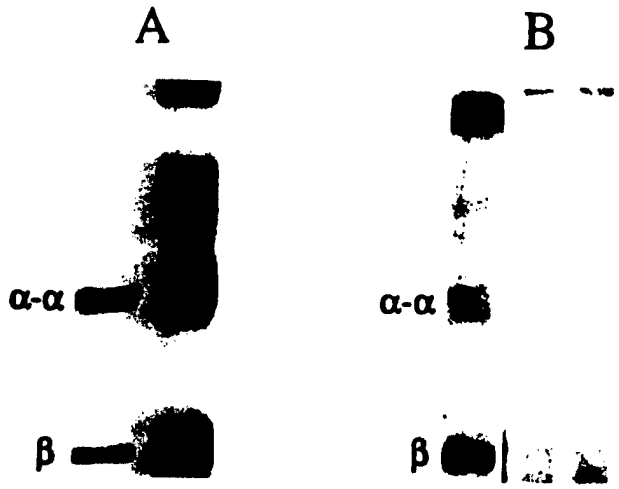


Figure 2 Photoaffinity labeling of Hb with ^{125}I-LPS-ASD. ^{125}I-LPS-ASD was incubated with $\alpha\alpha$Hb, photolyzed with UV light, and electrophoresed in SDS and 2-mercaptoethanol. Following electrophoresis, the gel was stained with Coomassie blue (A, left lane), dried, and subjected to autoradiography (A, right lane). Another photoaffinity-labeled $\alpha\alpha$Hb preparation from a separate experiment is shown (B, left lane), along with controls that consisted of an incubation mixture containing 100-fold excess unlabeled LPS as a blocking agent to demonstrate inhibition of specific binding (B, middle lane) and ^{125}I-LPS-ASD alone (B, right lane). (From Ref. 17.)

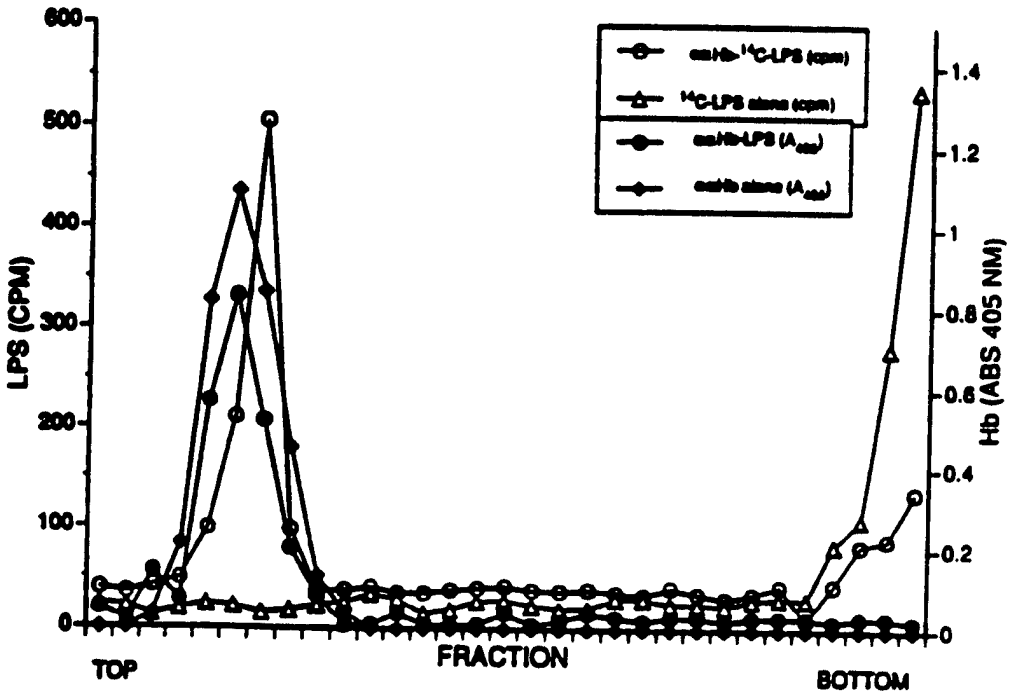


Figure 3 Sucrose density centrifugation of LPS-Hb. ^{14}C-LPS was incubated with $\alpha\alpha$Hb (100 mg/mL), and the mixture centrifuged through a 4–20% continuous sucrose gradient. Fractions of 0.4 mL were assayed for hemoglobin by absorbance at 405 nm (closed symbols) and for LPS by scintillation counting (open symbols). (From Ref. 17.)

formation of lower-density Hb-LPS complexes. Additional evidence of LPS dissociation was obtained by nondenaturing polyacrylamide gel electrophoresis, which demonstrated that LPS, when complexed with Hb, entered the gel and comigrated with Hb, whereas LPS alone remained within the stacking gel (Fig. 4). Ultrafiltration experiments demonstrated that LPS, which alone in aqueous solutions has a very high molecular weight (typically $\geq 10^6$ Da), cofiltered with Hb through 300- and 100-kDa membranes (Table 1). Whereas only 10–16% of LPS alone was filterable through the 300-kDa membrane and LPS alone was not filterable at all through the 100-kDa membrane, in the presence of Hb, 87–97% of LPS was filtered through the 300-kDa membrane and 64–72% through the 100-kDa membrane. Thus, these data provide further evidence that Hb greatly decreased the aggregate molecular weight of LPS.

Independent evidence that Hb is an LPS-binding protein has been provided by recent investigations that demonstrated binding of porcine Hb to the LPS of *Actinobacillus pleuropneumoniae* as well as binding to the surface of intact bacteria of this species (18). In addition, the LPS was shown to bind to

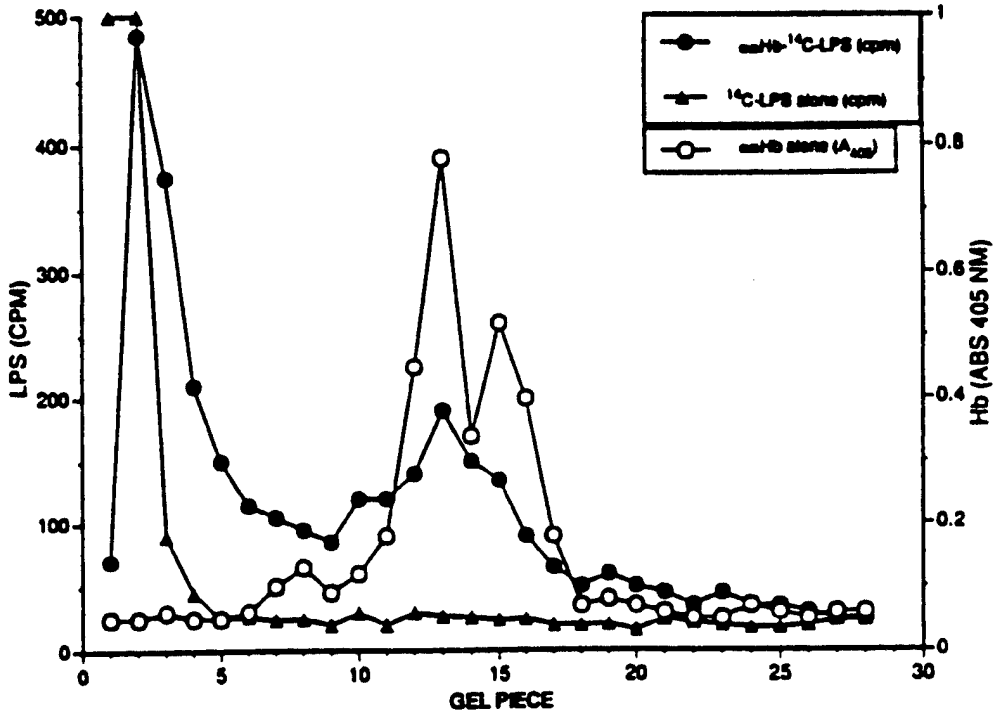


Figure 4 Electrophoresis of LPS and Hb. ^{14}C-LPS was incubated with $\alpha\alpha$Hb, and the $\alpha\alpha$Hb-LPS mixture or LPS alone was electrophoresed in polyacrylamide in the absence of SDS. ^{14}C-LPS was measured by scintillation counting of gel pieces (closed symbols), and $\alpha\alpha$Hb was monitored by absorbance at 405 nm (open circles). (From Ref. 17.)

Table 1 Ultrafiltration of *E. coli* O26:B6 and *P. mirabilis* S1959 LPS, Hb, and LPS-Hb Mixtures[a]

	E. coli LPS filtered (%)	*P. mirabilis* LPS filtered (%)	
	300-kDa[b] filter	100-kDa[b] filter	300-kDa[b] filter
LPS alone	10.2 ± 2.3	0	15.6 ± 5.6
$\alpha\alpha$Hb alone	0[c]	0	0
$\alpha\alpha$Hb + LPS	87.3 ± 8.0	63.6 ± 18.7	97.1 ± 1.5
$\alpha\alpha$HbCO alone	0	0	0
$\alpha\alpha$HbCO + LPS	89.3 ± 1.5	71.1 ± 4.0	90.9 ± 4.5
HbA_0 alone	0	0	0
HbA_0 + LPS	88.1 ± 3.7	71.6 ± 8.8	93.5 ± 8.6

[a]Each experiment was performed three times and the mean ±1 SD is shown. Percent of LPS filtered was determined with the chromogenic *Limulus* amebocyte lysate (LAL) test. LPS was quantified with reference to standard curves consisting of the respective LPS/protein mixture prior to filtration.

[b]Molecular weight cutoff of the filter.

[c]Lack of detectable LPS indicates that the starting preparations of Hb were endotoxin-free.

Source: From Ref. 17.

both the α and β chains of porcine Hb (18), confirming our observations that LPS binds to both the α and β chains of human Hb (17).

As described above, Hb alters several characteristics of LPS. Conversely, LPS can produce Hb denaturation, with production of methemoglobin and hemichromes (Fig. 5) (19). Degradation of Hb by LPS is time- (Fig. 6) and LPS concentration–dependent. There also are structural changes indicative of Hb oxidation, as demonstrated by circular dichroic analysis between 210 and 600 nm. However, there is no demonstrable change in the overall tertiary structure of the globin molecule (19).

A. Measurements of P_{50}

The oxygen affinity of Hb was measured in the absence and presence of LPS in order to evaluate the possible influence of LPS binding on Hb function (Table 2) (19). These measurements were made after a 2-hr incubation period, a time sufficient to result in Hb-LPS complex formation (20) but prior to the formation of substantial quantities of oxidized Hb species unable to bind oxygen. Because the two components of Hb-LPS complexes are of approximately equal concentration by weight and little unbound Hb is calculated to be present, 1 mg/mL Hb (16 μM) and 1 mg/mL of each LPS were utilized. Values of P_{50} for $\alpha\alpha$Hb (26.6 mmHg) and HBA_0 (9.6 mmHg) were slightly decreased by both smooth and rough LPSs (Table 2). Non-cross-linked cell-free HbA_0,

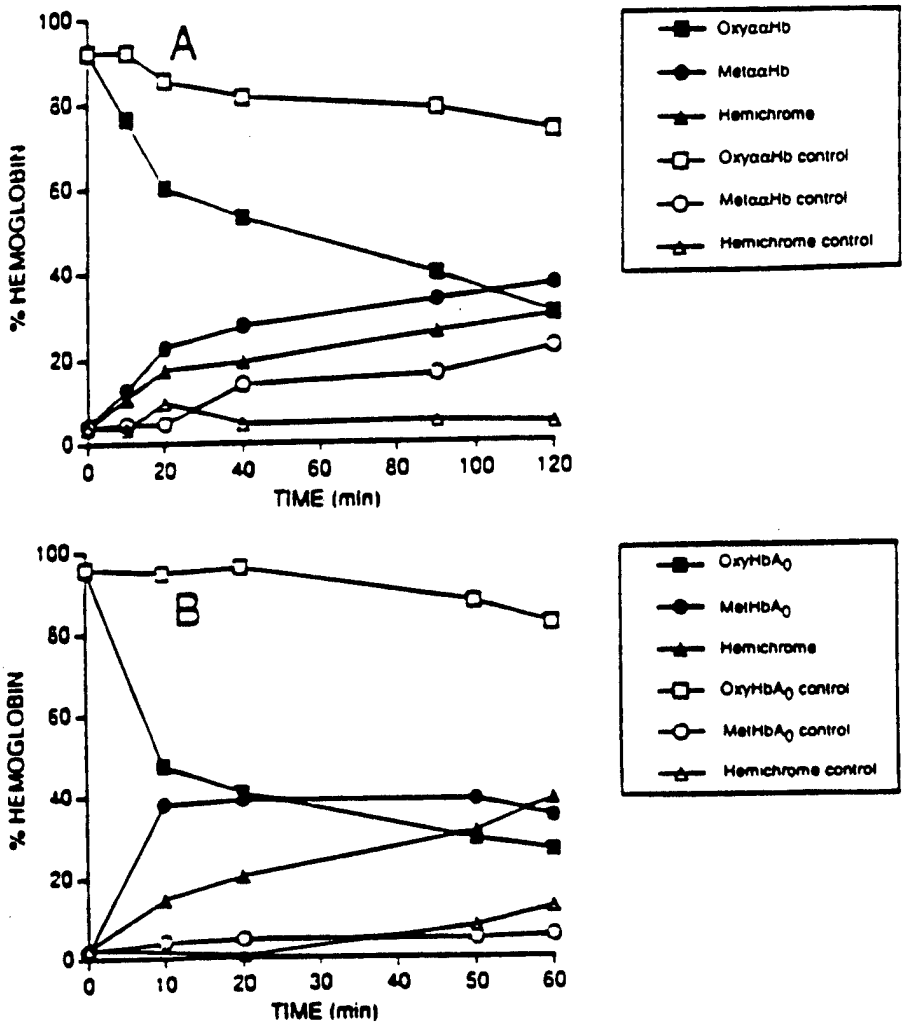


Figure 5 Time-dependent conversion of $\alpha\alpha$Hb (A) and HbA_0 (B) to metHb and hemichromes in the presence of *S. minnesota* 595 OH37 LPS (0.3 mg/mL and 0.8 mg/mL LPS incubated with $\alpha\alpha$Hb and HbA_0, respectively). Percentages of oxyHb, metHb, and hemichromes were determined according to the method of Winterbourn (Winterbourn C.C., *Methods Enzymol* 1990; 186:256–274). Open symbols, Hb alone; closed symbols, Hb + LPS. (From Ref. 19.)

which exhibited high oxygen affinity (P_{50} = 9.6 mmHg) similar to that measured with lysed whole blood (P_{50} = 10.0 mmHg; data not shown) best demonstrated the small trend toward higher oxygen affinity when in the presence of LPS (P_{50} = 7.3 mmHg in the presence of *S. minnesota* 595 OH37 LPS). Thus, there is little change in oxygen affinity of Hb when complexed to LPS.

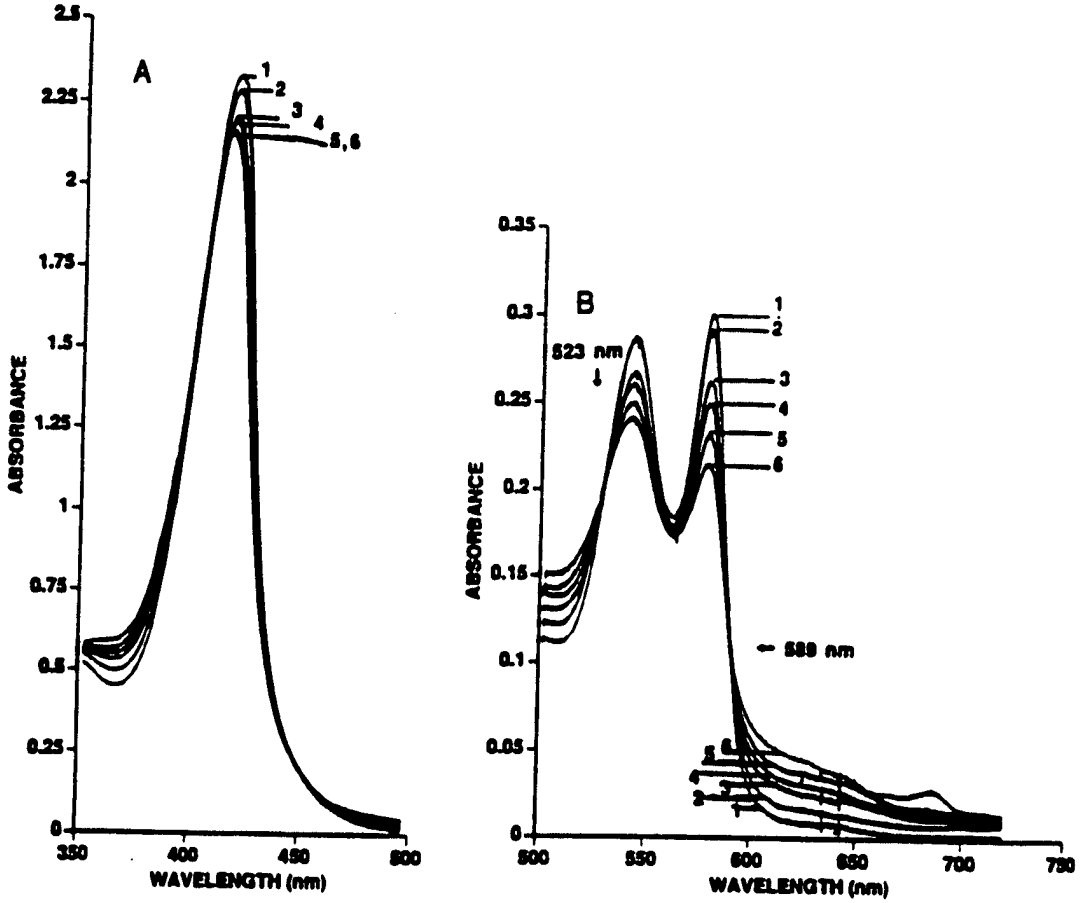


Figure 6 Time course of changes in the hemoglobin absorption spectrum in the presence of LPS. $\alpha\alpha$Hb (21 μM in PBS, pH 7.4) was incubated at 37°C in the presence of 0.3 mg/mL *S. minnesota* 595 OH37 LPS, and absorbance spectra in the Soret (A) and visible (B) regions of the Hb spectrum were obtained at various times of incubation. 1 = initial spectrum of $\alpha\alpha$Hb alone; 2 = 10 min; 3 = 20 min; 4 = 40 min; 5 = 90 min; 6 = 120 min. The sample cuvette contained Hb in PBS with or without LPS, and the reference cuvette contained PBS (for $\alpha\alpha$Hb spectra alone) or LPS alone (0.3 mg/mL in PBS) (for $\alpha\alpha$Hb-LPS mixture spectra). The arrows indicate the apparent isosbestic points. (From Ref. 19.)

III. EFFECT OF Hb ON LPS CLEARANCE IN VIVO

The clearance of LPS in rabbits was shown to be delayed in the presence of Hb (free Hb levels were 2 g/dL, which produced a 15% increase in total circulating Hb) compared to LPS clearance in animals given equivalent doses of human serum albumin (HSA) or NaCl (Fig. 7) (21). The intravascular retention of injected ^{125}I-LPS during the 30-min period analyzed was significantly longer

Table 2 P_{50} Values for Hb and Hb-LPS Complexes[a]

	P_{50}
$\alpha\alpha$Hb alone	26.6
$\alpha\alpha$Hb + LPS[b]	25.1
$\alpha\alpha$Hb + LPS[c]	25.6
HbA_0 alone	9.6
HbA_0 + LPS[d]	8.7
HbA_0 + LPS[e]	7.3

[a]Oxygen affinity measurements were obtained for cross-linked ($\alpha\alpha$Hb) and native (HbA_0) hemoglobins alone or in the presence of LPS after a 2-hr incubation at 37°C. Measurements were obtained prior to the production of oxidized Hb species. P_{50} was determined utilizing both smooth and rough LPSs.
[b]*P. mirabilis* 03 (smooth) LPS.
[c]*S. minnesota* Re 595 (rough) LPS.
[d]*E. coli* 026 (smooth) LPS.
[e]*S. minnesota* 595 OH37 (rough) LPS. Equal concentrations of Hb and LPS were utilized (each at 1 mg/mL prior to dilution in Hemox buffer).
Source: From Ref. 19.

in the LPS + Hb group than in the LPS + NaCl or LPS + HSA groups, especially during the initial 10 min. The intravascular half-life ($T_{1/2}$) of LPS in the LPS + NaCl control, LPS + HSA control, and LPS + Hb groups was 2.8, 4.0, and 4.9 min; the area under the curve was 1,369 ± 483, 1,594 ± 360, and 1,731 ± 481 (ng/mL × min, mean ± SD); and the total body clearance was 24.7 ± 9.2, 20.1 ± 5.4, and 18.9 ± 6.0 (mL/min, mean ± SD), respectively. The proportion of LPS associated with blood cells was very small at the initial 1-min time period and decreased even further during the 30-min period analyzed. Over 96% of injected LPS was associated with the cell-free plasma, with 51–54% of LPS in the apoprotein fraction at the initial time point and 35–37% in the high-density lipoprotein (HDL) fraction. The proportion of LPS increased significantly in the HDL fraction and decreased significantly in apoproteins during the 30 min period analyzed. However, there were no differences between the three groups (21,22). The liver was the main distri-

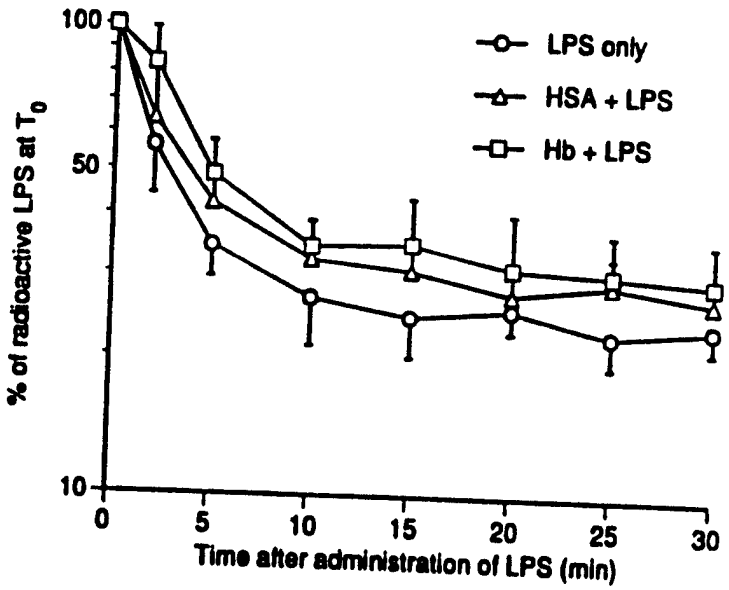


Figure 7 Intravascular clearance of ^{125}I-LPS after intravenous injection into rabbits: LPS only (○), LPS administered immediately following a 10-min infusion of HSA (△), or LPS following hemoglobin (Hb)(□). The numbers of animals in each group were 6, 6, and 5, respectively. Values represent the mean percent of the level of radioactive LPS in whole blood at T_0 ± SD. (From Ref. 21.)

bution site (74%) of injected LPS among the six organs evaluated (liver, kidney, lung, spleen, adrenal, and heart). In the Hb group, the accumulation of ^{125}I-LPS in the spleen was significantly lower than in the HSA group. The synergism of the in vivo toxicity reported for LPS and Hb may be due in part to the decreased rate of intravascular clearance of endotoxin.

IV. DEMONSTRATION THAT Hb ENHANCES THE BIOLOGICAL ACTIVITY OF LPS

A. Limulus Amoebocyte Lysate Activation

The effect of Hb on the biological activity of LPS was initially investigated using *Limulus* amoebocyte lysate (LAL), the most sensitive in vitro assay for LPS. A preparation from the blood cells of the horseshoe crab *Limulus polyphemus*, LAL contains an LPS-activated coagulation cascade that is sensitive to pg/mL concentrations of LPS. Each of a variety of LPS preparations obtained from *Proteus mirabilis* and spiked into solutions of LPS-free Hb demonstrated greatly increased activation of LAL, using a chromogenic endpoint, in comparison to identical concentrations of LPS assayed in saline (Fig. 8) (20).

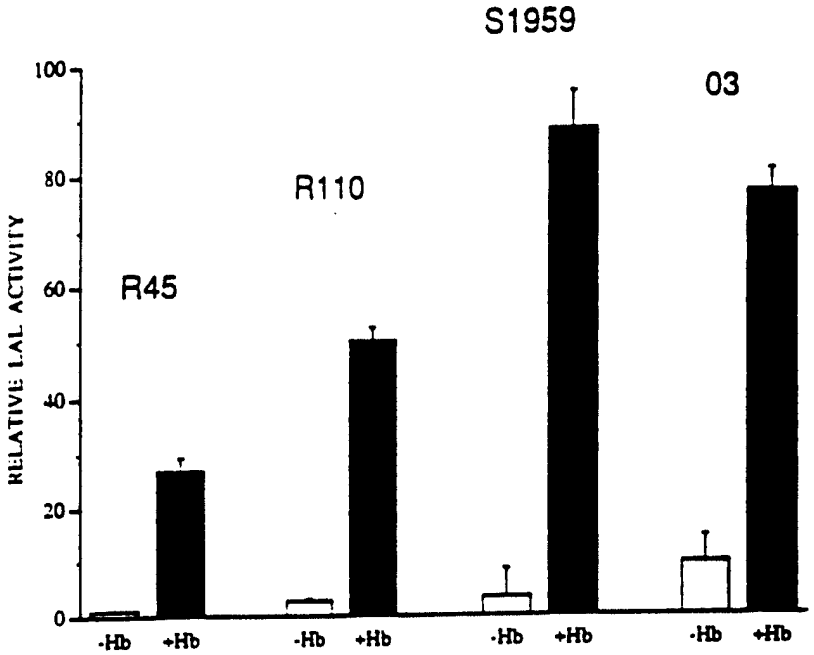


Figure 8 Enhancement by hemoglobin of the activation of *Limulus* amoebocyte lysate (LAL) by *Proteus* LPS. The LAL reactivities of LPS (500ng/mL) in the absence (−Hb) or presence (+Hb) of $\alpha\alpha$ cross-linked hemoglobin (1 mg/mL) were determined with the chromogenic LAL assay. To determine relative LAL activities, a standard curve of *P. mirabilis* R45 LPS was prepared, which related absorbance to LPS concentration. Using this standard curve, the absorbance for each sample (LPS alone or LPS-Hb) was converted into the equivalent R45 LPS concentration. 500 ng/mL R45 LPS was assigned a relative LAL activity of 1. Samples were assayed with eight replicates, and results are expressed as the mean ± 1 S.D. (From Ref. 20.)

Similar results were obtained with endotoxin obtained from *Salmonella minnesota* 595 and with purified lipid A (20). Furthermore, all three Hb preparations tested ($\alpha\alpha$Hb, HbA_0, and $\alpha\alpha$HbCO [carbon monoxy $\alpha\alpha$Hb]) produced enhanced activation of LAL over a wide range of LPS concentrations (Fig. 9). The enhancement by each Hb of LAL activation was also demonstrated with the gelation LAL test and was shown to be dependent on protein concentration (Fig. 10). The biological activity of LPS was enhanced >1000-fold at the concentrations of Hb that would be achieved in vivo for purposes of resuscitation. Pertinently, similar Hb concentrations have occasionally been detected in plasma following hemolysis associated with endotoxemia. These results are of great interest, because LAL activation is an excellent model for the intravascular coagulation commonly seen in humans during endotoxemia and repeatedly described during infusion of hemoglobin solutions in animals.

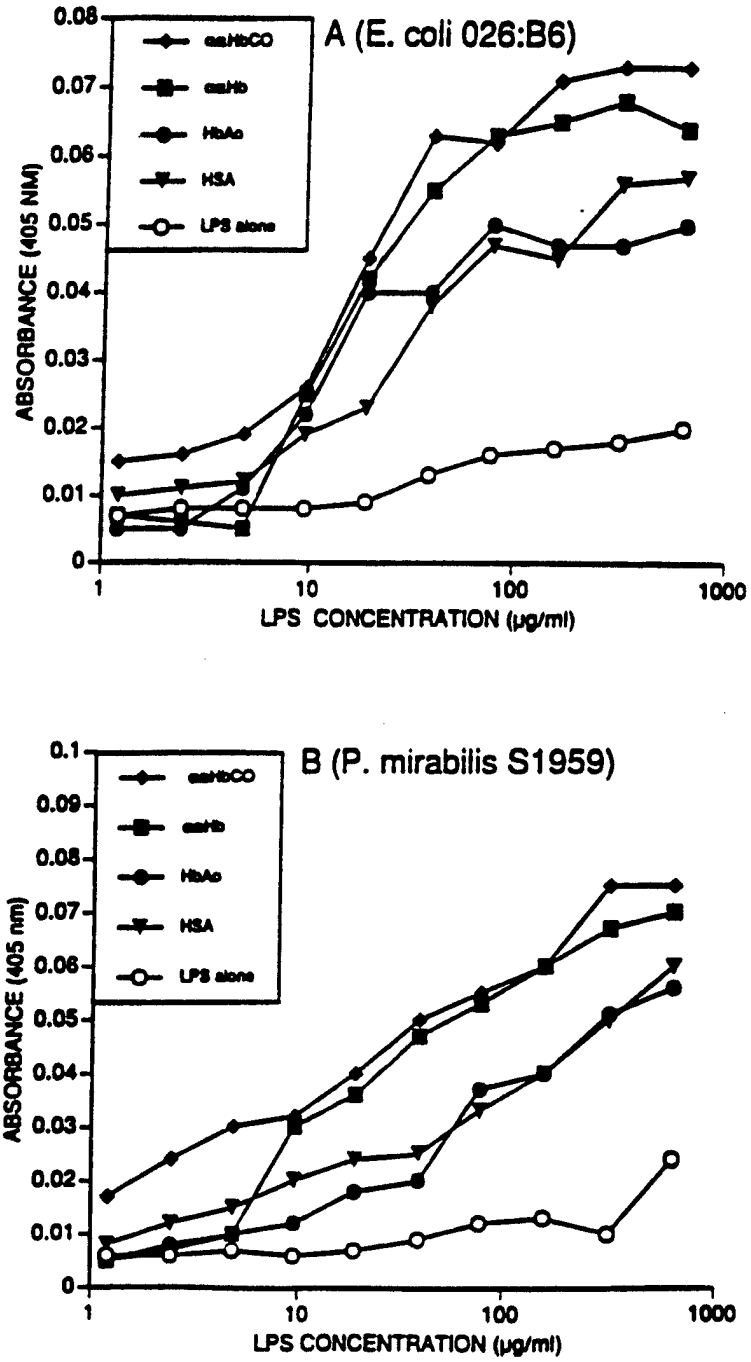


Figure 9 Enhancement of LPS activation of *Limulus* amoebocyte lysate (LAL) by Hb. Dilutions of *E. coli* O26:B6 LPS (A) or *P. mirabilis* S1959 LPS (B) in $\alpha\alpha$HbCO (◆), $\alpha\alpha$Hb (■), HbA_0 (●), HSA (▼), or NaCl (○) were assayed with the chromogenic LAL test. Absorbances at 405 nm were measured at 5 min. All protein concentrations were 1 mg/mL. Incubations were performed in triplicate, and the mean is shown. (From Ref. 17.)

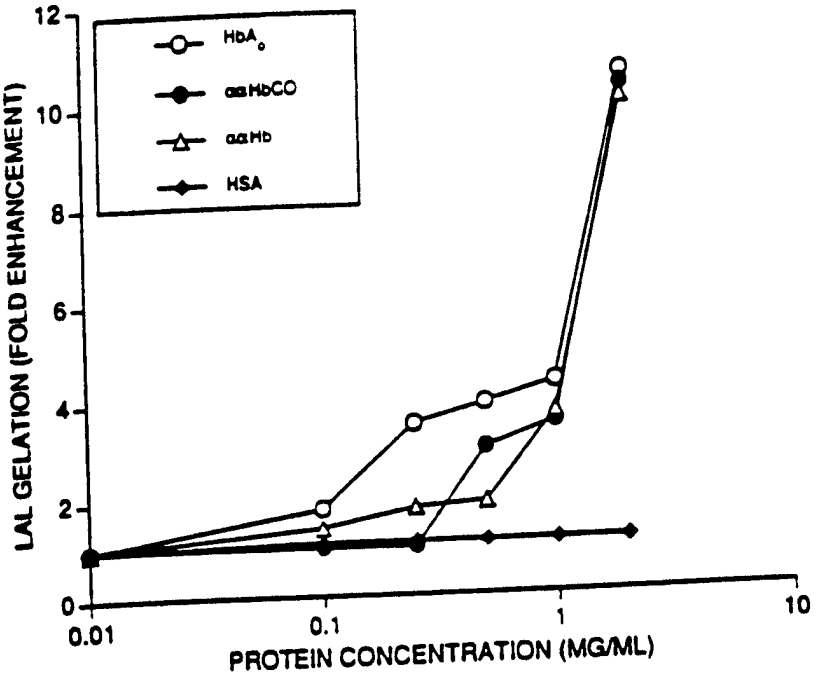


Figure 10 Enhancement of LPS activation of *Limulus* amoebocyte lysate (LAL) by Hb. Mixtures of LPS (100 pg/mL *E. coli* O26:B6) and HbA_0 (○), ααHbCO (●), ααHb (△), or HSA (◆) were assayed with LAL, using gelation as the endpoint. Protein concentrations ranged from 0.01–2 mg/mL. Enhancement of activation of LAL was calculated by comparison of the gelation time of each mixture to the gelation times for LPS solutions in 0.9% NaCl. 100 pg/mL LPS in 0.9% NaCl solution gelled *Limulus* amoebocyte lysate in 2.5 hr. Similar results were obtained from each of three independent experiments. (From Ref. 17.)

In order to further establish the generalized nature of the Hb enhancement effect, we studied the effect of ααHb on biological activities of several other LPSs, including LPSs from different bacterial species. Prominent and identical extents of enhancement by both ααHb and ααHbCO in the LAL assay were shown with three defined salts of *E. coli* 026:B6 (smooth LPS)—i.e., the calcium, sodium, and triethylamine forms—suggesting that the specific cations bound to LPS did not influence the Hb enhancement process. Enhancement of LPS biological activity also was demonstrated with a smooth *Salmonella* LPS (*S. abortus equi*) and a rough *E. coli* LPS (Re F515) but was not observed with nontoxic *Rhodobacter spheroides*, *Rhodobacter capsulatus*, and *Rhodopseudomonas viridis* LPSs.

Many of the LPS preparations studied had poor aqueous solubility and were visually turbid (especially *S. minnesota* 595 LPS, lipid A, and monophos-

phoryl lipid A; and *P. mirabilis* R110). Enhancement of LPS biological activity by Hb was a prominent feature of some of these LPSs and their partial structures, suggesting that a possible mechanism for the Hb enhancement effect was via increased LPS solubility. Therefore, we compared turbidity and the LAL biological activity of these LPSs in the absence and presence of Hb (20). With increasing concentrations of $\alpha\alpha$Hb, *P. mirabilis* R110 and *S. minnesota* 595 LPS each demonstrated a concomitant progressive decrease in turbidity and increase in LAL biological activity (Fig. 11).

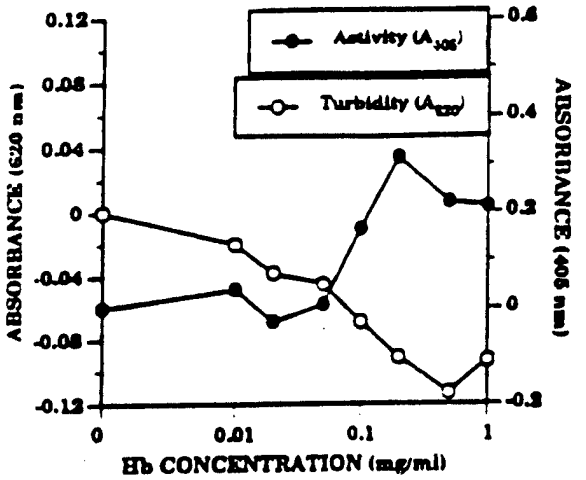
To further demonstrate the effect of Hb on the physical state of LPS, electron microscopic studies were performed. In the absence of Hb, *S. minnesota* (Re) 595 LPS was highly aggregated and consisted of variable ribbon-like, mesh-like, and/or membrane-like structures, with the largest dimensions greater than 1 μm (Fig. 12). However, in the presence of HbA_0, marked disaggregation of all of the highly aggregated LPS structures was demonstrated, with production of discoidal 5–20 nm particles (Fig. 13) (23). Similar results were shown with LPS from *E. coli* (Re) F515.

B. Tissue Factor Production

To further investigate the ability of Hb to modify LPS-activated coagulation, we evaluated the effect of Hb on LPS stimulation of peripheral blood mononuclear cell procoagulant activity (i.e., tissue factor, TF). This is another coagulation-based assay for LPS activity that is quantitative (as is the LAL assay) and is known to correlate well with LPS activity as determined by LAL. A Hb concentration–dependent enhancement of LPS-stimulated procoagulant activity in mononuclear cells was observed (Fig. 14) (24).

Since Hb has the ability to increase the production of TF by mononuclear cells, we reasoned that vascular endothelium might demonstrate a similar response. Cultured human umbilical vein endothelial cell (EC) monolayers were incubated with LPS in the presence and absence of Hb, and the generation of EC procoagulant activity (TF) was determined. LPS alone (0.01 to 10 μg/mL) caused a concentration-dependent increase in production of EC TF activity compared to the TF produced by unstimulated cells, while Hb resulted in augmented production of TF in response to LPS (Fig. 15) (25). Enhancement was not produced by IgG or human serum albumin (Fig. 16) (26). Enhancement was demonstrated with both native and cross-linked Hbs and was shown to be concentration-dependent between 0.1 and 100 mg/mL Hb. In both the presence and absence of Hb, the production of TF activity by LPS was completely inhibited by actinomycin D or cycloheximide, indicating a requirement for new protein synthesis. Elevated levels of TF protein in response to Hb-LPS, as assessed by an enzyme-linked immunosorbent assay (ELISA), were also demonstrated. Inhibition of nitric oxide synthesis, using *N*-monomethyl-L-arginine (NMMA),

P. mirabilis R110



S. minnesota 595

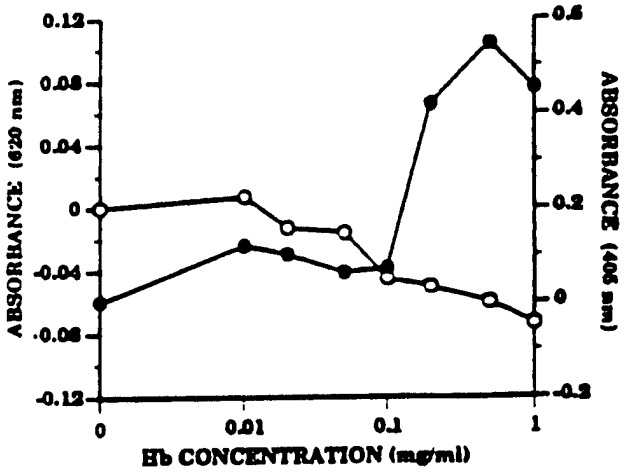


Figure 11 Turbidity and biological activity of LPS in the absence and presence of Hb. Various concentrations of $\alpha\alpha$ cross-linked Hb (from 0.01 to 1.0 mg/mL) were added to LPS (final concentration, 1 mg/mL) in microtiter plate wells and absorbances were measured. The turbidity of each LPS (absorbance at 620 nm) in the absence of Hb has been designated as 0; the change in absorbance induced by Hb is shown. Absorbances due to Hb have been subtracted. Actual baseline LPS absorbances were as follows: *P. mirabilis* R110, 0.21; *S. minnesota* R595, 0.12. LAL then was added to each well and chromogenic activities were determined at 405 nm. (From Ref. 20.)

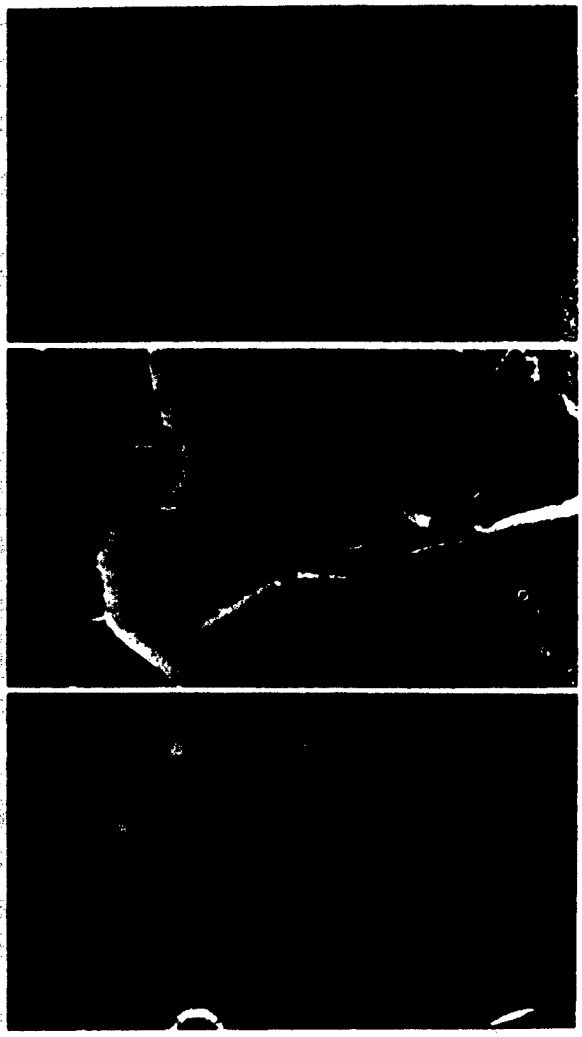


Figure 12 Top: Highly aggregated *S. minnesota* (Re) 595 LPS demonstrating primarily ribbon-like (arrows) and mesh-like (open arrows) structures (×60,000). Middle: Highly aggregated *S. minnesota* (Re) 595 LPS demonstrating primarily large membrane-like sheets (arrowheads) (×60,000). Bottom: High-power image (×180,000) of ribbon-like (arrows) and mesh-like (open arrow) Re LPS structures. (From Ref. 23.)

resulted in attenuated TF production (10–80% decrease of TF) by the EC in response to both LPS alone and Hb-LPS.

A possible mechanism for the enhancement by Hb of the stimulation of LPS-induced TF production was suggested by the demonstration that Hb increased the binding of LPS to EC (Fig. 17) (26). The increase in binding was related to the concentration of Hb. Furthermore, the increase in binding of LPS was produced only when LPS and Hb had been incubated for 30 min prior to

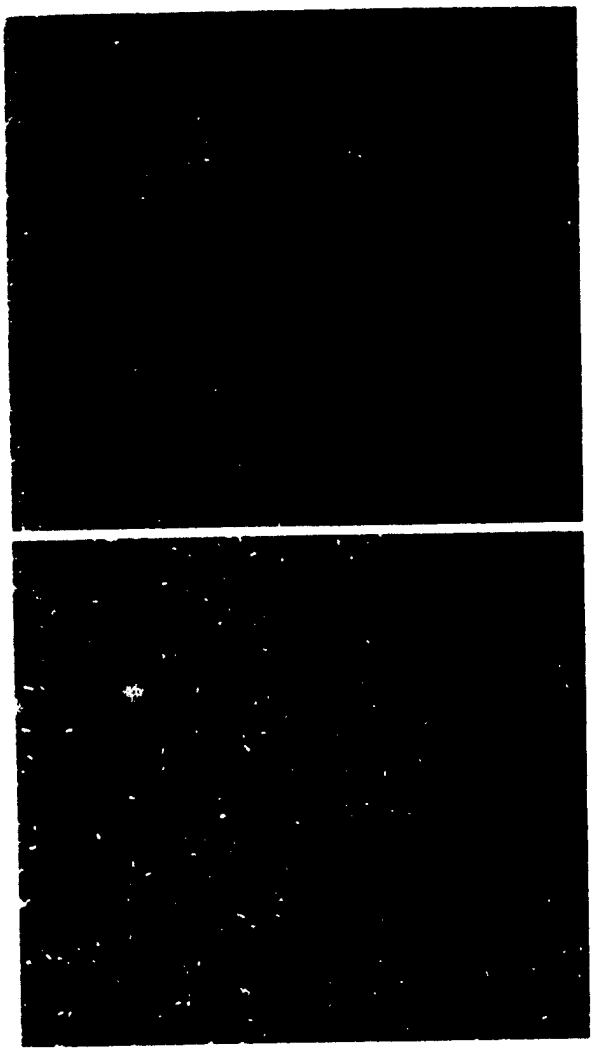


Figure 13 Top: Hb alone, consisting of uniform particles ~5.5 nm (×180,000). Bottom: Hb plus *S. minnesota* (Re) 595 LPS, following incubation at 37°C for 18 hr, showing disaggregation of LPS into small disk (arrow) and lens-shaped (arrowhead) particles of 5–20 nm (×180,000). None of the ribbon-like, mesh-like, or membrane-like structures of LPS remain after incubation with Hb. Very small Hb-LPS complexes are not distinguishable from Hb alone. (From Ref. 23.)

addition to the EC culture (Fig. 17). Increased binding was demonstrable both in serum-containing and serum-free medium as well as in plasma (Fig. 18). This indicated that soluble CD14 was not necessary for the binding of LPS under the conditions of these experiments. However, in the absence of serum, LPS binding to EC did not produce the biological response characterized by synthesis of TF.

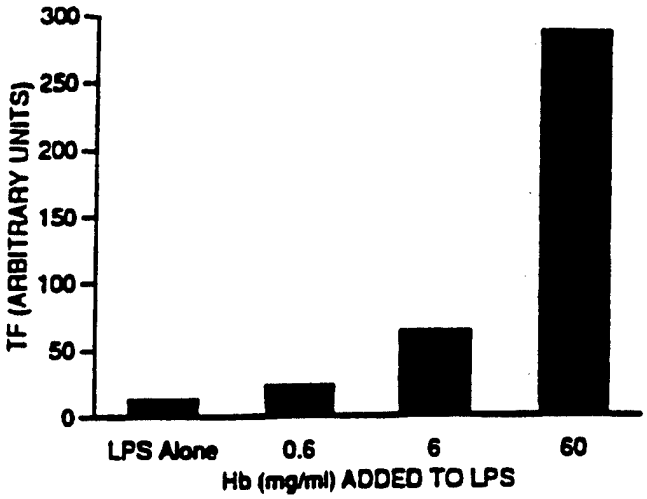


Figure 14 Tissue factor (TF) production by human mononuclear cells. Human mixed mononuclear cells were incubated with LPS in the presence of various concentrations of endotoxin-free Hb (0.6–60 mg/mL). TF generated by LPS alone and the Hb-LPS mixtures was determined following addition of citrated plasma and calcium (plasma recalcification assay). The contribution of the Hb alone (at each concentration, respectively) to the total TF generated by the mononuclear cells was subtracted from the measured total. (From Ref. 24.)

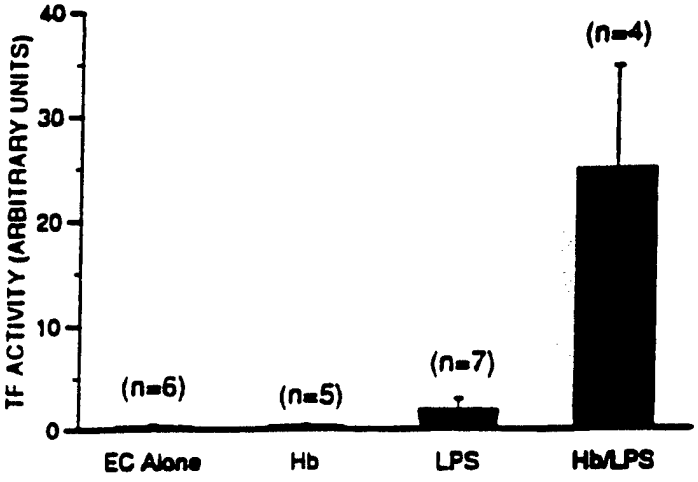


Figure 15 Effect of Hb on the production of human umbilical vein endothelial cell tissue factor (TF) in response to LPS. Cultured human endothelial cells (EC) were incubated with $\alpha\alpha$Hb alone, LPS alone, or LPS in the presence of $\alpha\alpha$Hb. TF activities were then determined with the plasma recalcification assay. (From Ref. 25.)

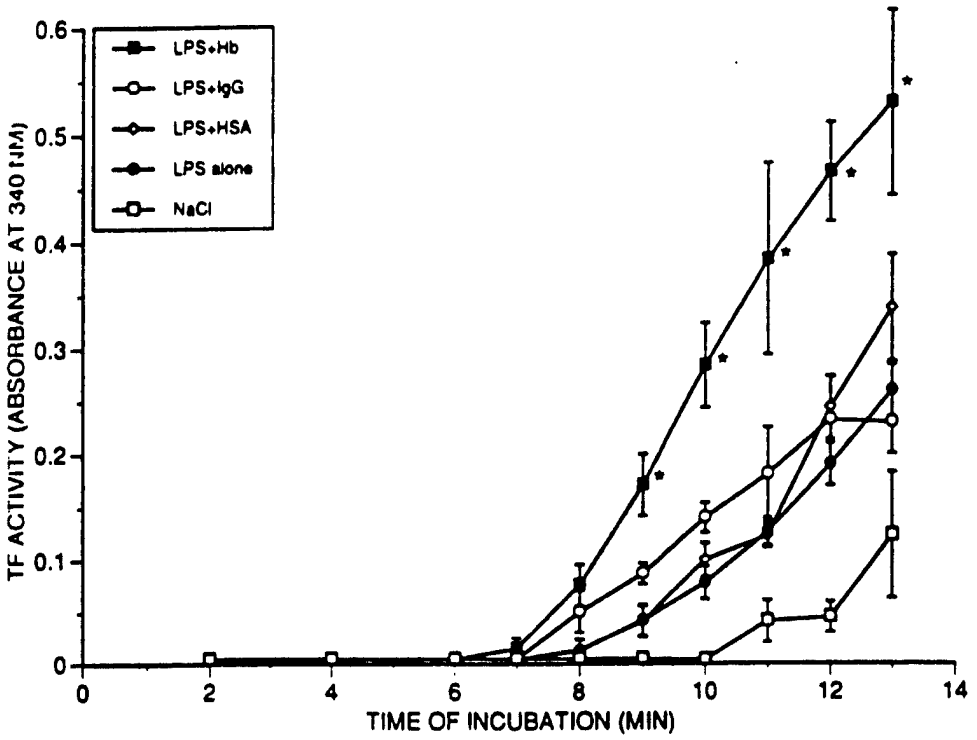


Figure 16 Effect of proteins on endothelial cell tissue factor (TF) activity. LPS was preincubated with Hb, HSA, or IgG; LPS alone or the LPS-protein mixtures were then added to human umbilical vein endothelial cells (EC) in medium containing 4% bovine serum. After 1 hr, the EC were washed, freeze-thawed, and sonicated; the plasma recalcification assay for TF was then performed. TF activity was assessed by the rate of increase in absorbance at 340 nm. Mean ± SD absorbances from 12 replicate wells are presented. $*p < 0.01$ increase versus LPS alone (Student's t-test). (From Ref. 26.)

C. Platelet Adherence to Endothelial Cells

Because of the critical role of the vascular endothelium in promoting pathological hemostatic responses to LPS in vivo (LPS transforms the endothelium from an anticoagulant surface to a procoagulant surface), we also examined whether Hb modified LPS-induced platelet adherence to endothelial cells (EC). Cultured human EC monolayers were incubated with LPS, in the presence and absence of HbA_0, and the binding of radiolabeled human platelets was examined. LPS alone resulted in slightly increased binding of human platelets to EC in culture (20% increase compared to platelet binding in the absence of LPS), and Hb-LPS complexes further increased platelet binding to EC (35% increase com-

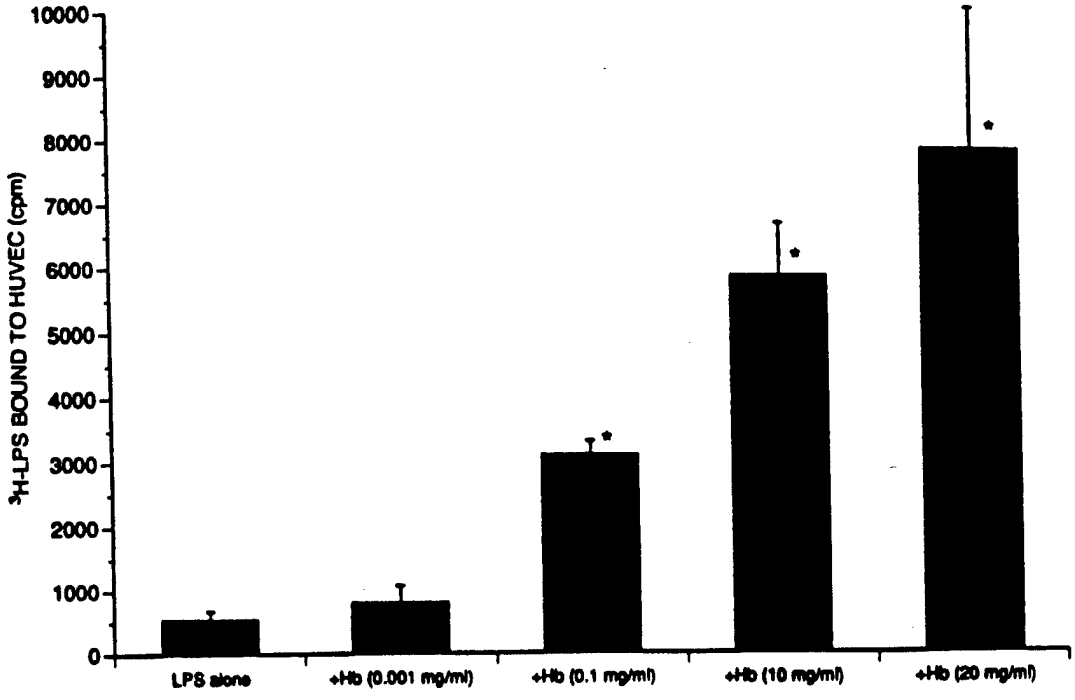


Figure 17 Effect of Hb on binding of LPS to human umbilical vein endothelial cells (HUVEC). ^{3}H-LPS was preincubated with various concentrations of Hb, and ^{3}H-LPS alone or the LPS-Hb mixtures were then added to HUVEC in medium containing 4% bovine serum. After 30 min, bound LPS was determined by scintillation counting. Binding assays were performed in two replicate wells per condition. Means and ranges are presented. $*p < 0.01$ increase versus LPS alone (Student's t-test). (From Ref. 26.)

pared to platelet binding in the control without LPS or Hb). Incubation of the EC with Hb alone resulted in a slight decrease in platelet binding.

D. Complement Activation

Enhancement by Hb of the biological activity of LPS in the activation of a proteolytic coagulation cascade in LAL suggested that there may be an impact of Hb on the ability of LPS to activate other protease cascades. We studied whether formation of Hb-LPS complexes altered the ability of LPS to activate and fix complement (a process thought to contribute to the in vivo toxicity of Hb in animal studies). Addition of Hb had little or no effect on the intrinsic complement fixing abilities of eight smooth endotoxins, rough endotoxins, or endotoxin partial structures (27). At higher concentrations (>0.2 mg/mL), Hb (both HbA^0 and $\alpha\alpha$Hb) alone was also capable of fixing complement, in the absence of LPS, via the classical pathway of complement activation (27).

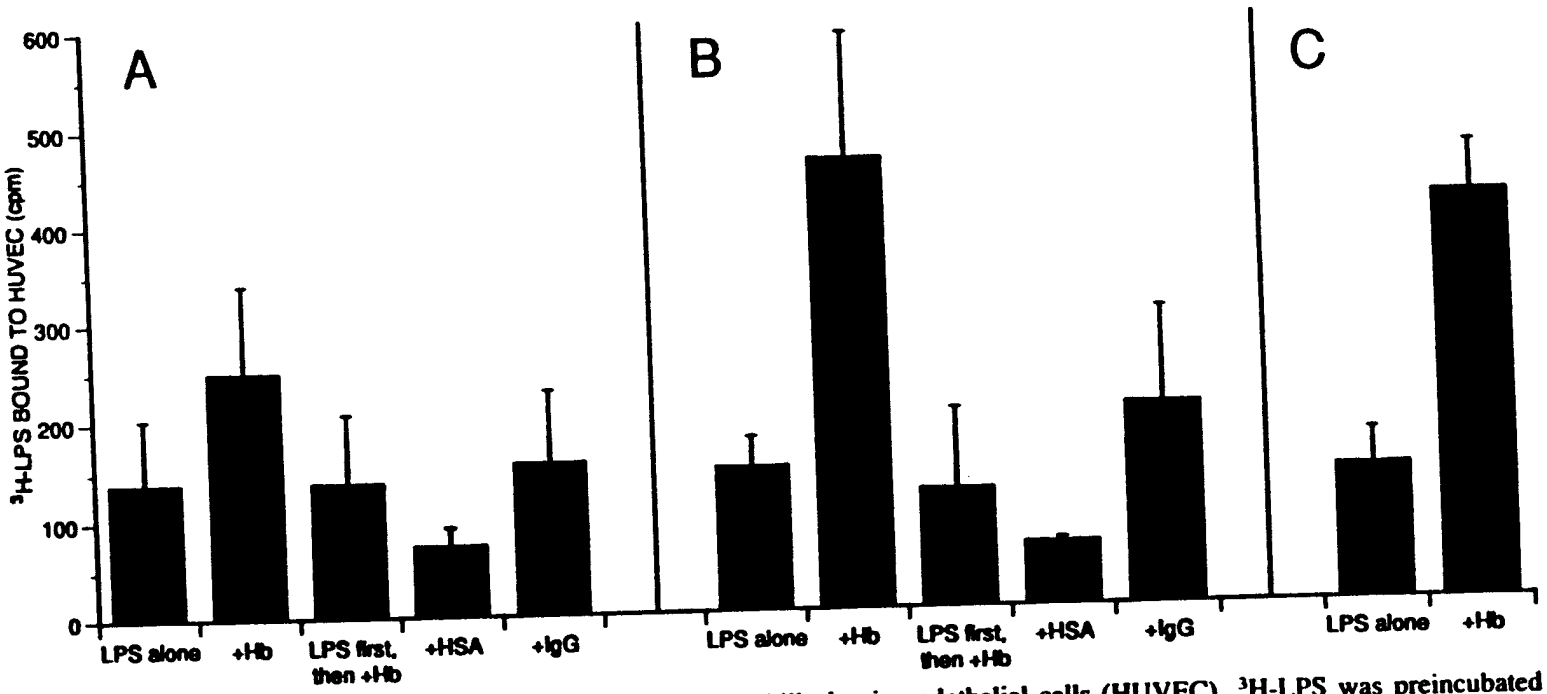


Figure 18 Effect of proteins on binding of LPS to human umbilical vein endothelial cells (HUVEC). ^{3}H-LPS was preincubated with Hb, HSA, or IgG (each 10 mg/mL, final concentration); ^{3}H-LPS alone or the LPS-protein mixtures were then added to HUVEC. After 30 min, bound LPS was determined by scintillation counting. In some wells, ^{3}H-LPS was added to the medium first, followed 5 min later by the addition of Hb. Binding assays were performed, with 6 replicate wells, in complete medium containing 4% bovine serum (A), in serum-free medium (B), or in 100% plasma (anticoagulated with hirudin) in the absence of medium (C). Means ± SD are presented. (From Ref. 26.)

E. Lethality in Mice

Because of the extensive in vitro data we obtained demonstrating the ability of Hb to enhance the biological activity of LPS, we initiated animal experiments to determine whether LPS-induced mortality was affected by the presence of hemoglobinemia. Mice were injected intraperitoneally with an LD_{50} dose of *E. coli* LPS (500 μg), and 8 hr later received an intravenous infusion of Hb (60 mg) sufficient to raise the blood Hb level by 4–5.5 g/dL. LPS-induced mortality was increased at several time points after Hb infusion (Fig. 19). Mortality in the Hb-treated mice was also noted many hours earlier than in mice that had received only LPS. Enhancement of mortality by Hb was observed over a range of doses of injected LPS. At a given endotoxin dose, enhancement of mortality was dependent on the dose of Hb administered. In the presence of endotoxemia, doses of Hb $\geq$45 mg resulted in increased mortality (Table 3). By itself, Hb caused no mortality, and mice that received Hb alone appeared completely normal throughout the study. Furthermore, Hb increased endotoxin-related mortality in mice whether it was infused intravenously prior to, coincident with, or subsequent to intraperitoneal endotoxin injection (Table 4).

Increased mortality in mice that had received LPS was observed for all preparations of Hb tested—i.e., $\alpha\alpha$Hb, HbA_0, $\beta\beta$Hb, and $\beta\beta/\alpha\alpha$Hb (Table 4).

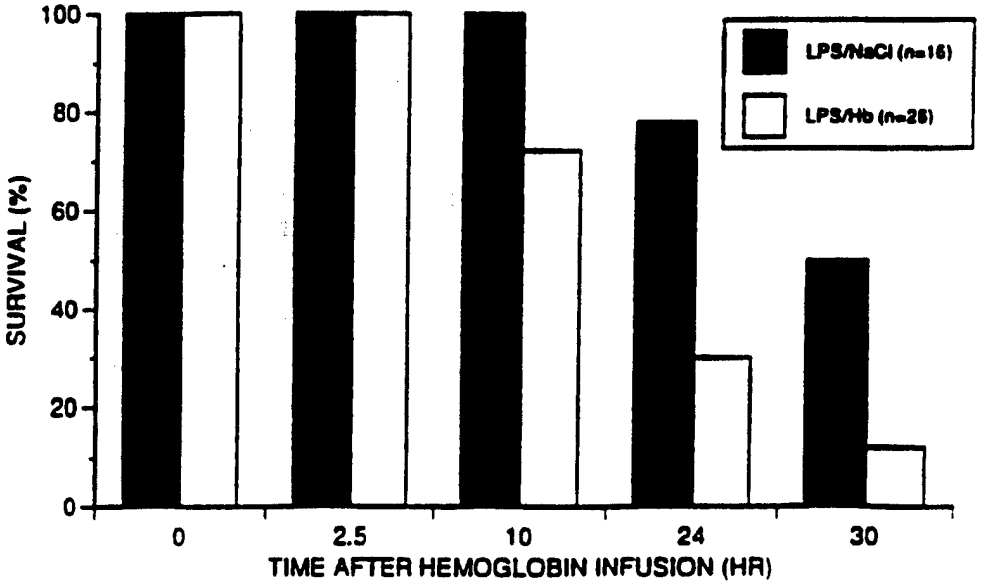


Figure 19 Effect of Hb on mortality from LPS. Mice were injected intraperitoneally with an LD_{50} dose of LPS (500 μg per animal), followed 8 hr later by intravenous infusion of 60 mg Hb per mouse (which generated a peak plasma Hb concentration of 4.0–5.5 g/dL) or 0.9% NaCl. Survival for 30 hr after Hb infusion is shown.

Table 3 LPS Lethality in Mice After the Administration of Various Doses of $\alpha\alpha$Hb[a]

	Survival at 48 hr (%)
LPS alone	59
LPS + 6 mg Hb	60
LPS + 11 mg Hb	60
LPS + 22 mg Hb	50
LPS + 45 mg Hb	12[b]
LPS + 60 mg Hb	7[b]

[a]Swiss Webster female mice (28–32 g) were injected intraperitoneally with 0.5 mg LPS (*E. coli* 055:B5 LPS, in sterile, pyrogen-free saline); 8–10 hr following LPS injection, the mice were infused by tail vein with either 0.6–0.8 mL saline or $\alpha\alpha$Hb in Ringer's acetate, pH 7.4 (doses of Hb ranged from 6–60 mg per mouse). Survival was monitored at 48 hr.

[b]$p < 0.01$ versus LPS alone (Fisher's exact p value).

Table 4 LPS Lethality in Mice in the Absence and Presence of $\alpha\alpha$Hb, $\beta\beta$Hb, HbA_0, or HSA[a]

	Survival at 24 hr (%)	Survival at 48 hr (%)
LPS alone ($n = 77$)	96	55
$\alpha\alpha$Hb alone ($n = 10$)	100	100
$\alpha\alpha$Hb + LPS (Hb before LPS) ($n = 11$)	54[b]	36
$\alpha\alpha$Hb + LPS (Hb with LPS) ($n = 28$)	32[b]	7[b]
$\alpha\alpha$Hb + LPS (Hb after LPS) ($n = 56$)	48[b]	7[b]
HbA_0 alone ($n = 14$)	100	100
HbA_0 + LPS (Hb with LPS) ($n = 19$)	32[b]	0[b]
$\beta\beta$Hb + LPS (Hb after LPS) ($n = 23$)	78[b]	9[b]
$\beta\beta/\alpha\alpha$Hb + LPS (Hb after LPS) ($n = 23$)	74[b]	9[b]
HSA + LPS (HSA with LPS) (n = 27)	100	48
HSA + LPS (HSA after LPS) ($n = 10$)	90	30

[a]Swiss Webster female mice (28–32 g) were injected intraperitoneally with saline or with 0.5 mg LPS (*E. coli* 055:B5 LPS, in sterile, pyrogen-free saline). Mice were also infused by tail vein with either 0.6–0.8 mL saline; Hb solutions in Ringer's acetate, pH 7.4 (60 mg per mouse); or human serum albumin (HSA, 60 mg/mL, pH 7.4, in saline with sodium bicarbonate). In various experiments, human $\alpha\alpha$Hb (DBBF cross-linked) was infused either 12 hr prior to, coincident with, or 8–10 hr subsequent to LPS. Bovine fumaryl $\beta\beta$ cross-linked Hb and human deca-sebacyl $\beta\beta/\alpha\alpha$ cross-linked Hb were infused 8–10 hr subsequent to LPS, and HbA_0 was infused coincident with LPS. HSA was infused either coincident with or 8–10 hr subsequent to LPS. Survival was monitored at 24 and 48 hr.

[b]$p < 0.01$ versus LPS alone (Fisher's exact p value).

This established that the effect of Hb on mortality was not limited to a single preparation of Hb nor was uniquely produced by the nature of the specific chemical cross-link. Other experiments suggested that an increased cytokine response and depressed reticuloendothelial cell function may have contributed to the enhanced mortality from LPS in the presence of Hb. Our observations are consistent with the previous reports indicating that the presence of free Hb in the circulation can compromise reticuloendothelial system function and increase susceptibility to bacterial infection (28–33).

V. SUMMARY AND CONCLUSIONS

Our data strongly support the conclusion that hemoglobin is an endotoxin-binding protein and that, as a result, LPS and Hb form complexes. The interaction between LPS and Hb alters each of the components of the Hb-LPS complex (Table 5). Importantly, the biological effects of LPS are enhanced and the UV spectrum of Hb is changed, consistent with methemoglobin formation and denaturation of the Hb molecule. Our observations indicate that the interaction between LPS and Hb results in marked disaggregation of the LPS macromolecule into smaller units that may approximate LPS monomers. The association between disaggregation and an increase in the biological activity of LPS is consistent with recent studies that have emphasized the relationship between the physical state of the LPS and biological activity (34–38). The spatial conformation of lipid A aggregates (e.g., lamellar versus non-lamellar) may play an important role in increasing the biological activity of LPS in aqueous biological systems. In addition, the relative concentrations of monomeric versus aggregated forms of LPS may also influence biological activity. However, this remains a controversial issue and may depend in part upon the concentration of LPS, its solubility, and the biological system utilized (39–41).

The interaction between Hb and LPS occurs with native HbA_0, $\alpha\alpha$Hb, or $\alpha\alpha$HbCO, the three forms of Hb that we have investigated in vitro. Further-

Table 5 Alterations of Hemoglobin (Hb) or Bacterial Endotoxin (LPS) Following Their Interaction

1. MW of LPS markedly decreased
2. Density of LPS decreased
3. LPS and Hb coelectrophoresed
4. Ethanol precipitability of Hb increased
5. Biological effects of LPS enhanced
6. Visible spectrum of Hb altered (MetHb and/or hemichromes formed)

more, a wide variety of LPSs have been shown to interact with Hb in the systems we have examined. Importantly, we have demonstrated that the administration of Hb also significantly increases the biological activity of LPS in vivo, as manifest by a marked increase in the mortality of mice that received both LPS and either native or cross-linked Hb. Therefore, our observations have potential relevance for the utilization of hemoglobin solutions as substitutes for RBC.

The development of noninfectious, nonantigenic, stable RBC substitutes for human use is of great importance in both civilian and military settings. Products presently under investigation include a variety of derivatized cell-free Hb preparations, perfluorocarbon emulsions, and encapsulated Hb preparations. Demonstrations of the safety of RBC substitutes, as well as their efficacy, have been identified as critically important by the Food and Drug Administration (42,43). Our data suggest that hemoglobin-based blood substitutes, which are currently undergoing clinical trials (44), may intensify the potentially fatal effects of the sepsis syndrome in patients with trauma, infection, or hypotension who receive hemoglobin for RBC replacement. Others have also recently expressed concern about the potential danger of administering hemoglobin-based RBC substitutes to patients with sepsis, ischemia, or shock (the latter two clinical conditions can predispose to the development of endotoxemia, even if endotoxin is not the precipitating cause of ischemia or hypotension) (45,46). Therefore, Hb should be administered to such patients with caution and thorough serial physiological observations performed in order to detect any worsening of signs or symptoms that may be attributable to endotoxemia and the sepsis syndrome. In this regard, it may be useful to measure endotoxin levels in the blood of these patients.

ACKNOWLEDGMENTS

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RED BLOOD CELL SUBSTITUTES

BASIC PRINCIPLES AND CLINICAL APPLICATIONS

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Limulus Antilipopolysaccharide Factor Prevents Mortality Late in the Course of Endotoxemia

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Limulus **antilipopolysaccharide factor (LALF) can neutralize bacterial endotoxin, but its ability to prevent mortality following prolonged endotoxemia is unknown. Mice were challenged with an LD_{50} dose of intraperitoneal *E. coli* lipopolysaccharide (LPS) and then received LALF at various times after administration of LPS. Survival at 72 h was significantly improved by the administration of LALF at 4, 10, and even 24 h after LPS (73%, 78%, and 65% survival, respectively, vs. 15% survival in controls). Following intravenous administration of LALF at either 10 or 24 h after LPS, plasma levels of biologically active LPS abruptly fell (>1000-fold lower than pre-LALF levels). Plasma LALF concentrations fell much more gradually in LPS-treated mice ($t_{1/2}$ = 120 min) than in control mice ($t_{1/2}$ = 2.5 min). In conclusion, LALF markedly decreased plasma concentrations of biologically active LPS and protected mice from lethality even when LALF was not administered until long after the onset of continuous endotoxemia.**

Lipopolysaccharide (LPS, bacterial endotoxin), a toxic component of the cell wall of gram-negative bacteria, is often responsible for initiation and perpetuation of the sepsis syndrome. Antisepsis treatment based on neutralization of LPS or LPS-mediated pathways has been a major focus of medical research (reviewed in [1]) because of the belief that early interruption of the sepsis cascade would provide the best chance for successful clinical intervention. A variety of systemically administered LPS-binding proteins have been evaluated for their clinical efficacy, with generally unimpressive results [2]. A major problem with antiendotoxin therapy is that, whereas endotoxemia can be intermittent and recurrent, administered LPS-binding proteins are typically cleared rapidly; consequently, LPS is only transiently bound and neutralized by these therapies. Prolonged protection against circulating LPS would presumably prove more useful.

A 12-kDa protein that binds LPS with high affinity (K_d ~50 n*M*) has been obtained both from the Japanese and North American horseshoe crabs, *Tachypleus tridentatus* and *Limulus polyphemus,* respectively [3–5]. Alternatively referred to as *Limulus* anti-LPS factor (LALF) or endotoxin neutralizing protein, this protein has been isolated from the membranes of amebocytes (the only type of circulating blood cell in these animals) and recently also has been obtained as a recombinant protein in yeast [6]. Substantial structural similarities, including a prominent amphipathic loop involved in LPS binding, exist between LALF from the horseshoe crab and two physiologically important mammalian LPS-binding proteins, the plasma protein LPS-binding protein and the leukocyte granule protein bactericidal/permeability-increasing protein (BPI) [7]. LALF binds the lipid A portion of a variety of chemically diverse LPSs, is bactericidal, and has been shown to neutralize biologic activities of LPS in several in vitro assays, including LPS-induced activation of limulus amebocyte lysate (LAL), splenocytes, and endothelial cells [8–10]. LALF also has been shown to be protective in several animal models of endotoxin challenge, when given temporally close to endotoxin [4, 6, 10–12] or bacterial challenge [13–18] (in all studies, LALF was administered from 30 min before to 60 min after challenge). The potential utility of LALF administered later in the course of endotoxin challenge, which has not been investigated, would be important because of the intermittent or recurrent nature (or both) of endotoxemia during sepsis, especially when the sepsis syndrome is well established at the time of diagnosis.

The model of endotoxemia produced by an intravenous bolus of LPS is convenient for demonstration of antiendotoxin effects in experimental treatment protocols but is not generally realistic as a model for human sepsis. Human endotoxemic sepsis typically originates either from an extravascular site of bacterial infection or from translocation of bacteria or LPS (or both) from the gastrointestinal tract secondary to gut ischemia or liver dysfunction. Therefore, sepsis in humans does not usually

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This study was approved by the Institutional Review Board for the care of animal subjects at the VA Medical Center, San Francisco. The investigators adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH publication no. 86-23, revised 1985).

Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the US Army.

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result from only a single intravenous bolus of LPS, which is relatively quickly cleared, but rather is often accompanied by the presence of endotoxemia, which may be intermittent or protracted. Animal models of sepsis in which circulating LPS is derived from an extravascular site (e.g., the peritoneum) more closely resemble the usual human clinical situation, and therefore provide a more useful preclinical evaluation of a potential antiendotoxin therapy. Such an animal model has been utilized extensively in our laboratory to determine the ability of an infused protein (i.e., human hemoglobin) to influence the course of endotoxemia [19]. Sepsis models in which circulating LPS is derived from an extravascular source of gram-negative bacteria also produce an animal model of sepsis with prolonged endotoxemia [15]. In the current report, we present evidence from mice that LALF may provide a much wider window of protection against LPS than has been previously recognized.

Materials and Methods

Reagents. Purified native LALF was prepared in the laboratory of one of the investigators (N.R.W.), as previously described [4]. LALF was endotoxin-free, stored lyophilized at −20°C, reconstituted in 0.1 *M* Tris, pH 9.5, and then adjusted to pH 7.4 in endotoxin-free PBS prior to administration. *Escherichia coli* O55:B5 (smooth) LPS was obtained from Difco Laboratories (Detroit), suspended in isotonic saline, and sonicated for 3 min before administration. ^{3}H (biosynthetically radiolabeled) *E. coli* K12 (Rb) LPS, obtained from List Biological Laboratories (Campbell, CA), was similarly reconstituted.

Animal sepsis model (endotoxin challenge). Swiss-Webster female mice (28–30 g; Simonsen Laboratories, Gilroy, CA) were injected intraperitoneally with an LD_{50} at 48 h dose (500 μg/mouse) of *E. coli* O55:B5 LPS. Previous studies with this model have demonstrated that LPS is detectable in plasma with the LAL assay within 30 min of administration. A broad plateau of high LPS levels, produced between 4 and 10 h after administration, then decreases gradually over the subsequent 24–32 h. Lower but detectable levels of LPS are present in plasma as long as 72 h after administration [19]. In this model, a peak of detectable tumor necrosis factor (TNF) occurs 1–1.5 h after intraperitoneal administration of LPS, considerably in advance of the maximum plasma LPS levels. LALF or saline alone (for control animals) was administered in a total volume of 0.8–0.9 mL by tail vein over ~1 min. Mice received one of three doses of LALF: 0.7 mg/mouse (23.8 mg/kg; high dose), 0.2 mg/mouse (6.8 mg/kg; intermediate dose), or 0.07 mg/mouse (2.4 mg/kg; low dose). LALF was administered at one of three times after intraperitoneal LPS injection: 4 h after LPS (at the beginning of the broad period of maximum plasma LPS levels), 12 h after LPS (after several hours of maximal endotoxemia), or 24 h after LPS (after most of the LPS absorbed from the peritoneal cavity had been cleared from the blood, and after which animals began to die from sepsis). Mortality was monitored for 72 h after LPS administration.

In separate control experiments to distinguish between possible mechanisms of LALF effects on endotoxemia, mice were injected intraperitoneally with [^{3}H]LPS, with or without unlabeled *E. coli* O55:B5 LPS, followed by intravenous LALF as described above. Plasma was prepared, and scintillation counting was performed, after samples were diluted 10-fold in fluor (formula A-989; NEN Research Products, Boston), in an analytical liquid scintillation system (Tracor Analytic, Elk Grove Village, IL). Recovery of spiked isotope in preliminary experiments demonstrated >90% detection of added radioactivity.

Endotoxin assay. Plasma (anticoagulated with EDTA) was prepared from 70-μL blood samples obtained with capillary tubes from the retroorbital sinus. LPS concentrations were determined in diluted plasma (usually 1:10,000 in pyrogen-free water, rarely 1:100,000 for very high LPS concentrations), without an extraction step, using a chromogenic LAL assay (Endospecy; Seikagaku Kogyo, Tokyo), as described previously [19].

LALF ELISA. LALF concentrations in plasma were determined by ELISA, using rabbit antibody to native LALF prepared in the laboratory of one of the investigators (N.R.W.). ELISA was performed essentially as described [20] except that the buffer used for adsorbing LALF was 0.1 *M* CAPS (3-cyclohexylamino-1-propane sulfonic acid), pH 10.2. Samples of plasma were diluted 1:100 in this buffer before addition to the microtiter plate wells. LALF binding to the plates and subsequent detection were found to be excellent without necessity of a capture antibody. Standard curves similarly were obtained from LALF spiked into plasma, then diluted 100-fold before assay. To ensure that the presence of LPS in blood did not interfere with the binding of LALF to the wells, standard curves of LALF spiked into undiluted normal plasma and into plasma containing 100 μg/mL LPS were compared; these standard curves were essentially identical. For the determinations of LALF clearance in vivo, the standard curve was constructed by performing the assay on a series of LALF concentrations from 0 to 250 ng/mL (final concentrations after 1:100 dilution) in normal mouse plasma. With the procedure utilizing a 1:100 dilution of plasma, ~1.6 μg/mL LALF in the original samples was detectable.

Statistical analyses. Survival between groups was compared using Fisher's exact test. Plasma LPS and LALF levels between groups were compared with the Mann-Whitney rank sum test.

Results

Survival. In a large control group of mice (n = 48) that received LPS only, mortality was progressive after 24 h, with 48% mortality at 48 h (figure 1, open bars). Mice that received high-dose LALF (0.7 mg/mouse) (figure 1, solid bars) 10 h after LPS injection had improved survival at 48 and 72 h compared with control animals (91% vs. 48% survival, respectively, at 48 h, $P < .01$; 78% vs. 15% survival, respectively, at 72 h, $P < .01$). The intermediate dose of LALF (0.2 mg/mouse) (figure 1, stippled bars) produced a trend toward improved survival at 48 h and clear evidence of efficacy compared with control at 72 h (44% vs. 15% survival, $P = .02$), whereas low-dose LALF (0.07 mg/mouse) was ineffective (figure 1, hatched bars). High-dose LALF (0.7 mg/mouse) also was protective compared with control when administered 4 h after LPS challenge (73% vs. 15% survival, respectively, at 72 h, $P < .01$) (figure 2), and surprisingly when infused 24 h after LPS

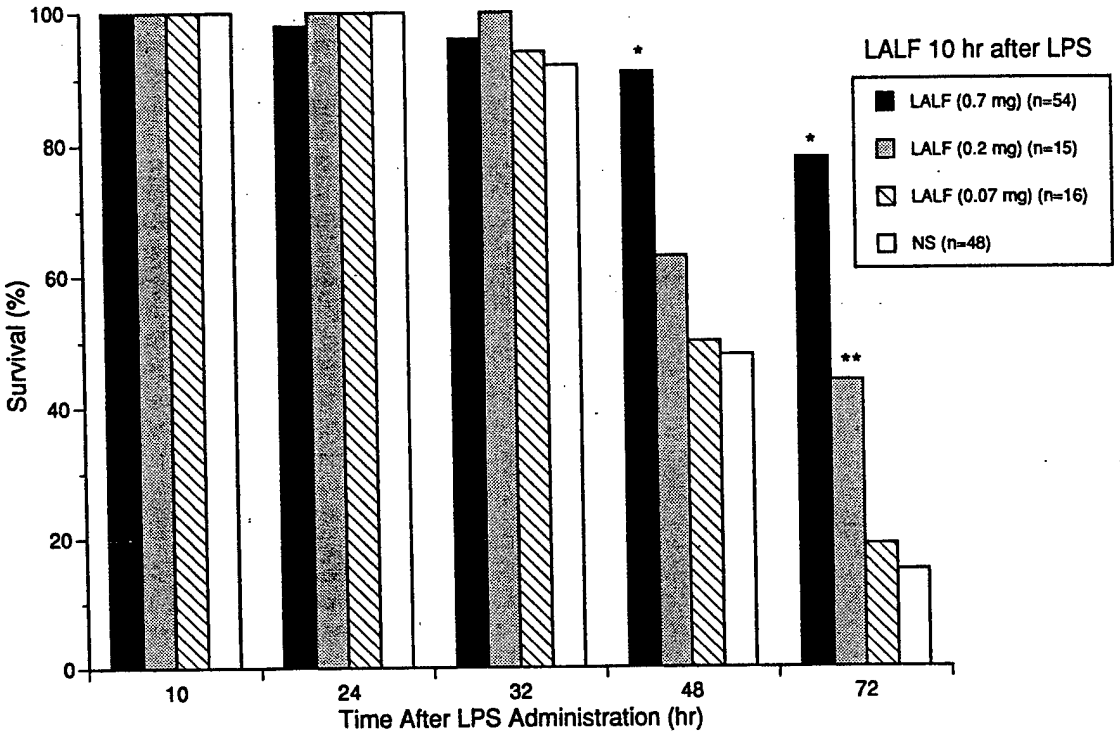

Figure 1. Survival after *Escherichia coli* LPS challenge: effect of LALF 10 h after LPS. Mice received LD_{50} intraperitoneal dose of *E. coli* LPS (500 μg) and, after 10 h, received intravenous high-dose LALF (solid bars), intermediate-dose LALF (stippled bars), low-dose LALF (hatched bars), or normal saline (NS, open bars). Compared with controls, which received saline, treatment with high-dose LALF improved survival at 48 and 72 h (* $P < .01$ vs. control, Fisher's exact test). Treatment with intermediate-dose LALF improved survival at 72 h (** $P = .02$ vs. control), whereas low-dose LALF had no effect on mortality.

(82% vs. 48% survival at 48 h, $P = .02$; 65% vs. 15% survival at 72 h, $P < .01$) (figure 3).

LPS plasma concentrations. Intraperitoneal injection of LPS resulted in prolonged endotoxemia (figure 4, open bars). The maximum concentration of biologically active LPS detected in plasma (~100 μg/mL at 4 h after LPS injection) indicated initial absorption from the peritoneum of at least ~20% of the injected dose. Plasma levels of biologically detectable LPS remained markedly elevated for many hours and then gradually declined. LPS concentrations were still high at

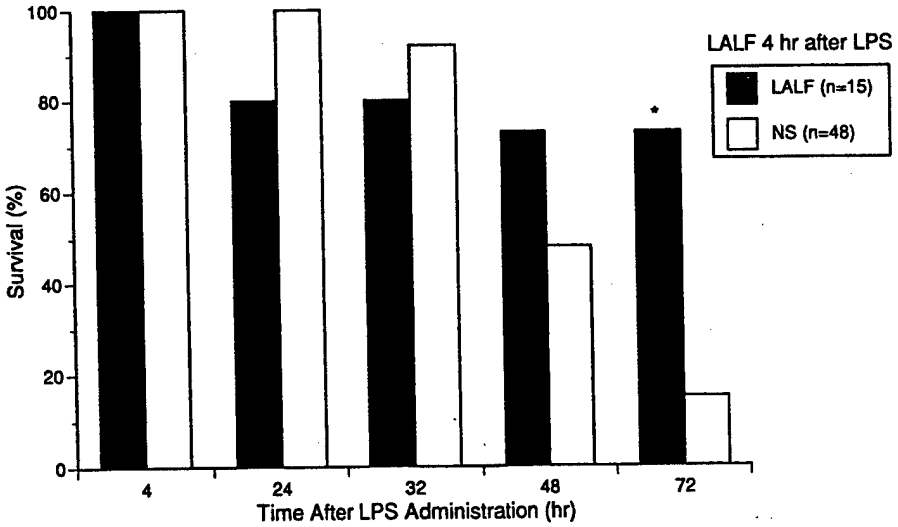

Figure 2. Survival after *Escherichia coli* LPS challenge: effect of LALF 4 h after LPS. Mice received LD_{50} intraperitoneal dose of *E. coli* LPS and, after 4 h, received intravenous high-dose LALF (0.7 mg, solid bars) or normal saline (NS, open bars). Compared with controls, which received saline, treatment with LALF improved survival at 72 h (* $P < .01$ vs. control, Fisher's exact test).

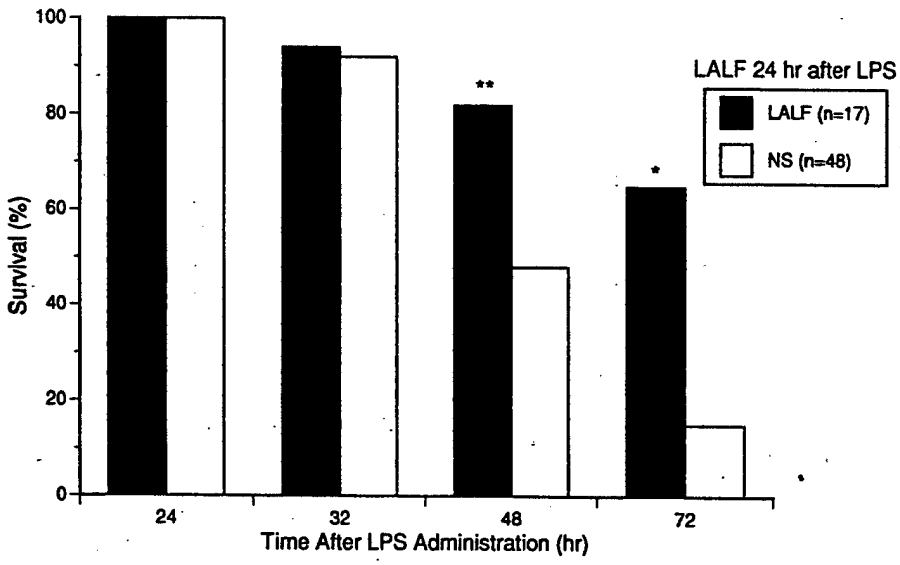

Figure 3. Survival after *Escherichia coli* LPS challenge: effect of LALF 24 h after LPS. Mice received LD_{50} intraperitoneal dose of *E. coli* LPS and, after 24 h, received intravenous high-dose LALF (0.7 mg, solid bars) or normal saline (NS, open bars). Compared with controls, which received saline, treatment with LALF improved survival at 48 h (** $P = .02$ vs. control, Fisher's exact test) and 72 h (* $P < .01$ vs. control).

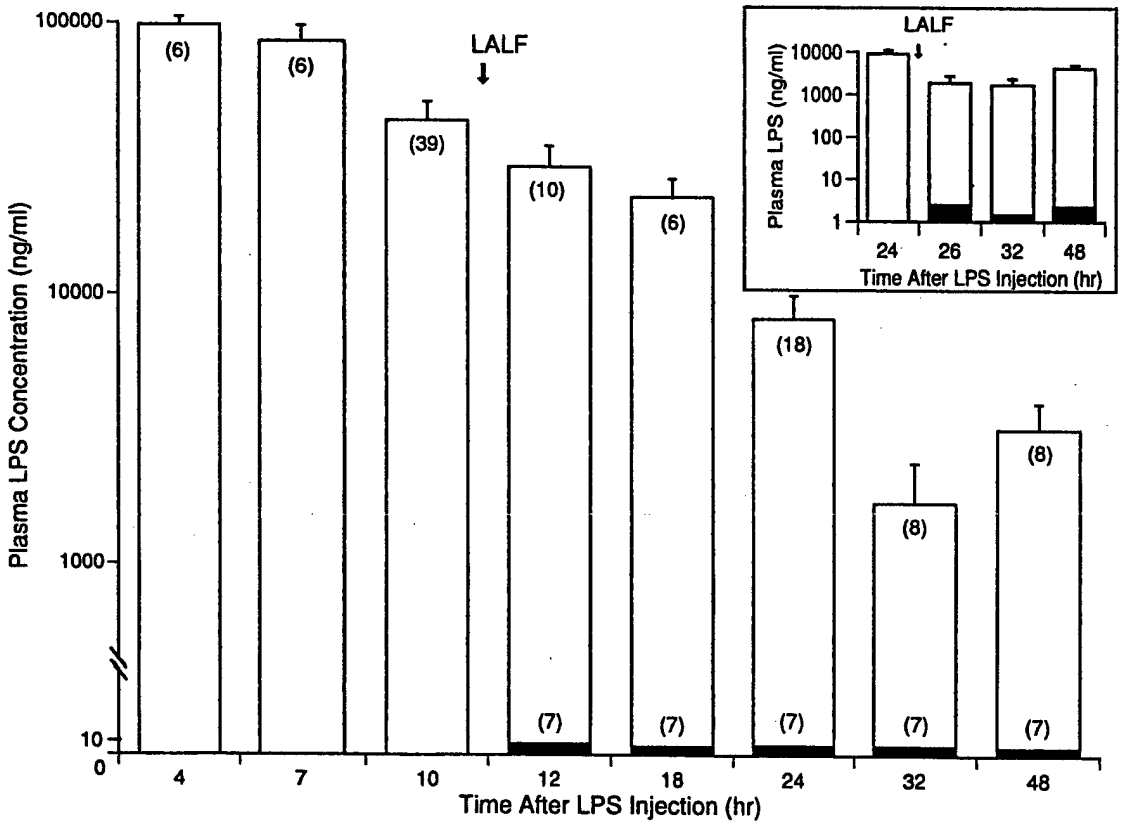

Figure 4. Mean plasma LPS levels after LPS challenge: effect of LALF. Mice received LD_{50} intraperitoneal dose of *Escherichia coli* LPS (500 μg), and plasma samples were assayed for LPS concentration during subsequent 48 h (open bars). Nos. in parentheses = no. of plasma samples tested. Mean ± SE is shown. High-dose LALF (0.7 mg/mouse) was administered intravenously to 7 mice 10 h after LPS injection (solid bars). Plasma obtained 2–38 h after LALF had LPS concentrations that had decreased ~1000-fold ($P < .01$ vs. pre-treatment LPS concentrations, Mann-Whitney rank sum test). LPS levels in control mice, which received LPS but not LALF, were not significantly changed between 10 and 12 h after LPS (<2-fold decrease in LPS concentrations). Inset: 7 additional mice received intraperitoneal LPS and then high-dose LALF 24 h after LPS (solid bars). Plasma obtained 2–24 h after LALF had LPS concentrations (mean ± SE is shown) that had decreased ~2,000-fold ($P < .01$ vs. pretreatment LPS concentrations, Mann-Whitney rank sum test). LPS levels in control mice, which had received LPS but not LALF, decreased only ~4-fold between 24 and 26 h after LPS.

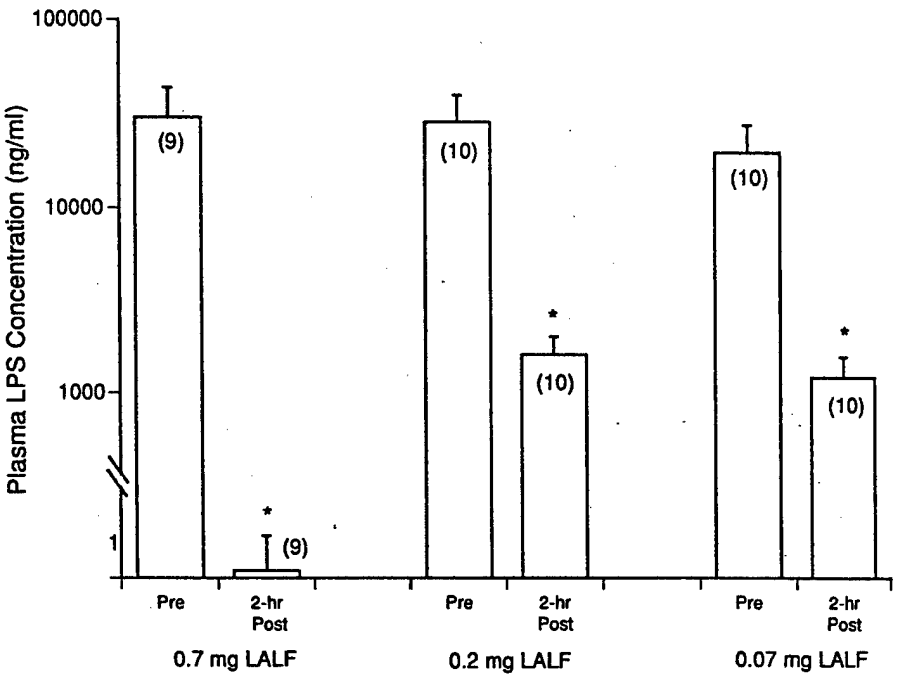

Figure 5. Mean plasma LPS levels after LPS challenge: effect of LALF dose. Mice received LD_{50} intraperitoneal dose of *Escherichia coli* LPS and, 10 h later, received high-dose (0.7 mg/mouse), intermediate-dose (0.2 mg/mouse), or low-dose (0.07 mg/mouse) intravenous LALF. Plasma obtained 2 h after high-dose LALF had LPS concentrations (mean ± SE is shown) that had decreased >1000-fold, while decreases in LPS concentrations after the intermediate and low doses were ~20-fold (* $P < .01$ vs. pretreatment LPS concentrations, Mann-Whitney rank sum test).

24 h after administration (the late time selected for testing the effect of LALF) and remained so at 48 h. High-dose LALF (0.7 mg) administered 10 h after LPS resulted in a marked decrease in plasma concentration of biologically active LPS (>1000-fold) that persisted during the subsequent 36 h (figure 4, closed bars). Injection of high-dose LALF 24 h after LPS also produced a similarly marked decrease in LPS biologic activity (~2000-fold) (figure 4, inset). Intermediate (0.2 mg/mouse) and low (0.07 mg/mouse) doses of LALF administered 10 h after LPS also produced decreases in concentrations of biologically active LPS (17- to 20-fold) (figure 5), although these lower doses of LALF produced lesser effects than high-dose LALF.

LALF plasma concentrations. LALF administered by intravenous infusion into normal mice had rapid initial clearance from the circulation, with a $t_{1/2}$ of 2.5 min. After the initial clearance of ~90% of infused LALF, measurable but low concentrations of LALF (~5 μg/mL) remained for many hours. In contrast to the rapid initial clearance of LALF in normal mice, LALF clearance in mice that had received intraperitoneal LPS 11 h before LALF infusion was much slower ($t_{1/2}$ = 120 min), and as long as 20 h after infusion, substantial plasma LALF concentrations (>10 μg/mL) were observed. In mice that received LALF 24 h (rather than 11 h) after intraperitoneal LPS, LALF clearance was even slower ($t_{1/2}$ = ~7 h) and LALF remained detectable (~10 μg/mL) at 96 h after administration.

To determine whether the ability of intravenous LALF to decrease plasma LPS involved clearance of LALF into peritoneal fluid and the subsequent neutralization of LPS prior to its entrance into the circulation, samples of peritoneal fluid were obtained 2 h after intravenous infusion of LALF into normal mice. There was no detectable LALF in these samples, indicating that the markedly decreased levels of plasma LPS observed after intravenous LALF resulted from an effect of LALF on circulating LPS rather than on LPS in the peritoneal cavity.

We used radiolabeled LPS in studies to determine whether the ability of intravenous LALF to decrease plasma LPS concentrations represented enhanced clearance of LPS or neutralization of the biologic activity of LPS remaining in the circulation. [^{3}H]LPS (2 μg, 10^6 cpm), with or without 500 μg of unlabeled LPS, was injected intraperitoneally (3 mice/group). At 4 h after LPS administration, LALF was injected intravenously (5–50 μg of LALF in animals that had received only [^{3}H]LPS and 0.7 mg of LALF in animals that had received [^{3}H]LPS + unlabeled LPS). At 2 h after LALF administration, LPS levels were similar to pre-LALF levels (50%–80%), indicating that the 3- to 4-log decrease in LPS concentration by LAL assay represented neutralization of LPS biologic activity rather than rapid clearance of LPS.

Discussion

Experimental approaches to the treatment of sepsis include methods based on neutralizing or enhancing clearance (or both) of compounds that initiate the sepsis syndrome (e.g., bacterial LPS) or on modulating the host's responses to sepsis (e.g., the inflammatory cytokine cascade). LALF, which is an example of the former approach, has produced encouraging results in a number of animal studies, suggesting the potential clinical use of this protein in the setting of endotoxemia [4, 6, 10–12].

In addition to confirming the previously reported findings of others, that LALF can be protective in animal models of

endotoxemic sepsis, our studies unexpectedly demonstrated that LALF markedly improved survival in mice when it was administered 10 and even 24 h after injection of LPS into the peritoneal cavity. To our knowledge, this late protection by infusion of an LPS-binding agent is unprecedented. Efficacy of LALF was demonstrated even though plasma LPS levels had been high from 4 h after LPS injection; previous investigations with this animal model have demonstrated that LPS can be detected in plasma within 30 min of administration [19].

Therefore, the exciting finding of our studies is that LALF was effective in treating endotoxemia without a requirement for the neutralization of LPS early in the septic process. This has not been demonstrated previously using intravenous LPS models in which there appeared to be a requirement for administering LALF in close temporal proximity to the LPS infusion (e.g., within ~30 min of LPS) in order to demonstrate efficacy [11, 12]. An important difference between the intraperitoneal and intravenous administration of LPS is that endotoxemia develops more slowly and then subsequently is much more prolonged in our model compared to models using intravenous LPS. It is possible that the route of administration of LPS (intraperitoneally vs. intravenously) is critical for the demonstration of efficacy. Alternatively, it is possible that species differences account for the discrepant findings between studies.

Although the ability of LALF to dramatically decrease circulating plasma levels of biologically detectable LPS has now been clearly demonstrated by ourselves and several others [11, 12, 15], it is not certain that this mechanism always accounts for the protective effect of LALF. In the current study, although the intermediate and low doses of LALF produced comparable decreases in levels of circulating endotoxin, only the intermediate dose improved survival. In two studies of *E. coli* sepsis from other laboratories, in which intraperitoneal injection of bacteria was followed by antibiotic treatment (thus generating endotoxemia without bacteremia), LALF improved survival without substantially altering circulating LPS levels [16, 17]. A similar dissociation between effects of an endotoxin-binding protein on survival vs. circulating LPS levels has been demonstrated with polymyxin B; protection against lethality was afforded for *E. coli* LPS but not for *Neisseria meningitidis* LPS, although plasma LPS levels were not decreased by polymyxin B in either experiment [21].

In our experimental model of endotoxemia, in which there was improved survival in mice when LALF was administered 24 h after LPS, plasma LPS levels had decreased ~90% below maximum before infusion of LALF, indicating that extensive clearance of LPS had already occurred. Our results suggest the possibility that LALF may affect the biologic activity of cleared (i.e., extravascular) LPS, although we cannot rule out the possibility that LALF is beneficial in experimental endotoxemia by mechanisms other than neutralization of LPS.

Our demonstration of LALF efficacy many hours after LPS administration is of further interest with regard to the presumed contribution of cytokines, such as plasma TNF, to the pathologic consequences of endotoxemia. The abrupt spike of TNF in the circulation, in response to intraperitoneal LPS, occurs 1.5 h after injection in this model (unpublished data); therefore, protection by LALF against LPS lethality is demonstrable even when LALF is administered many hours after initiation of the systemic inflammatory response syndrome. Others have demonstrated that, after LALF administration, there was no correlation between TNF levels and survival in individual animals [11]. A similar dissociation between clinical outcome and TNF levels also has been observed with the LPS-binding protein BPI, which was shown to decrease LPS levels and improve survival in rat models of endotoxic shock [22] and hemorrhagic shock [23] but with either no change or even a paradoxical increase in TNF levels.

Another unexpected finding from our studies was the observation that LALF circulated longer in mice with endotoxemia (substantial blood levels of LALF were detected at the termination of the experiments, 20 h after infusion) than in control mice. It has been proposed that LALF is cleared from the circulation into the reticuloendothelial cell system (RES), in particular hepatic Kupffer cells [6]. Because LPS may cause RES depression [24], it is possible that LALF clearance in endotoxemic mice is slowed due to this systemic effect of LPS. Alternatively, LALF may be differently distributed in the blood of endotoxemic and normal mice. Since most LPS in blood is associated with lipoproteins [25], the clearance of LALF in endotoxemic mice may be determined by the clearance of LALF-LPS-lipoprotein complexes. In contrast, it is likely that injected LALF circulates as a soluble protein in mice that have not received LPS.

Our animal model of endotoxemia is similar to human clinical conditions in which there is a tissue pool of bacteria and LPS (e.g., peritonitis), and the prolonged presence of circulating endotoxin is similar to the typical time course of clinical sepsis in humans. The ability of LALF to be protective at widely disparate times after induction of endotoxemia is a novel finding for an endotoxin-binding/neutralizing protein. Importantly, LALF has been shown to be beneficial, even when administered very late in the course of endotoxemia. If LALF that persists in the circulation remains capable of binding additional LPS, the observation that intravascular persistence of LALF was prolonged in endotoxemic animals suggests that LALF may prove beneficial in sepsis during which there may be several intermittent episodes of endotoxemia.

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HEMOGLOBIN INFUSION AUGMENTS THE TUMOR NECROSIS FACTOR RESPONSE TO BACTERIAL ENDOTOXIN (LIPOPOLYSACCHARIDE) IN MICE

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Key Words: Bacterial lipopolysaccharide; endotoxin; hemoglobin; tumor necrosis factor; reticuloendothelial cell system; monocyte/macrophage system; animal model

ABSTRACT

Objective: To determine whether cell-free hemoglobin (Hb) augments the inflammatory cascade, as detected by production of tumor necrosis factor (TNF) elicited by bacterial endotoxin (LPS).

Design: In vivo and ex vivo study, using a mouse model of sepsis.

Setting: Animal research facility

Subjects: Female Swiss Webster mice

Interventions: For the in vivo experiments, an LD50 dose (500 μg) of E. coli lipopolysaccharide (LPS) was injected intraperitoneally into mice. Cell-free, crosslinked hemoglobin (60 mg/mouse) or saline was administered intravenously 10 hr prior to or coincident with LPS. For the ex vivo experiments, hemoglobin (60 mg/mouse) or saline was administered intravenously to mice, and 10 hr later hepatic Kupffer cells, peripheral blood mononuclear cells, or peritoneal macrophages were isolated.

Measurements and Main Results: Intravenous infusion of hemoglobin either 10 hr prior to or coincident with intraperitoneal LPS resulted in a peak of plasma TNF that was greater than in control mice given LPS only. Cultured Kupffer cells, isolated from mice that had received Hb in vivo 10 hr prior to cell collection, produced more TNF in response to LPS in vitro than cells from normal mice. A trend toward greater TNF production in vitro by peripheral blood mononuclear cells obtained from Hb-treated mice also was observed. Enhanced sensitivity to LPS was not observed with cultured peritoneal macrophages from mice which had received hemoglobin.

Conclusions: Intravenous hemoglobin increased the sensitivity of hepatic macrophages to subsequent stimulation by LPS. This effect may contribute to the increased mortality which we have observed in animals that have received both LPS and hemoglobin.

Concern over safety and availability of transfused erythrocytes has been a driving force for the development of alternative oxygen carrying solutions (1-3). Many of the transfusion solutions currently being developed and clinically tested are based on acellular hemoglobin (cell-free hemoglobin, Hb). Chemical and/or genetic modifications of Hb can be utilized to produce oxygen carrying solutions with appropriate oxygen binding affinities and that can circulate in the bloodstream for substantial periods of time. Cell-free Hb solutions have been demonstrated to have efficacy in animal models of hemorrhage, and appear likely to be similarly useful for resuscitation in humans. However, experimental studies have demonstrated potential toxicities that can arise from the interaction of cell-free Hb and infectious agents. Specifically, the lethality of gram-negative bacterial infection is increased by hemolysis (4), administration of iron (5,6), or the infusion of Hb (7-9). Therefore, infusion of Hb may increase mortality in patients who have pre-existing sepsis, or who become septic after receiving Hb.

In addition to the well-known deleterious interaction between Hb and bacteria, recent studies from our laboratory (10) have demonstrated that Hb similarly increases lethality from bacterial endotoxin (lipopolysaccharide, LPS), the toxic component of the gram-negative cell wall responsible for many of the pathologic processes that occur during sepsis. A variety of chemically-modified Hb preparations, as well as unmodified, purified native HbA_0, have been shown to markedly enhance LPS-mediated mortality. In an extensive series of studies utilizing Hb crosslinked between the α chains with bis(3,5-dibromosalicyl) fumarate (DBBF), an LD50 (48 hr mortality) dose of intraperitoneal E. coli LPS (in the absence of Hb) resulted in ~90% mortality in association with the intravenous infusion of Hb (10). The mechanism of this deleterious interaction between Hb and LPS has been a major focus of investigation in our laboratory. In vitro, Hb has been shown to bind and markedly increase several biological activities of LPS (11-15).

Therefore, we have hypothesized that Hb similarly may be able to augment LPS activity in vivo.

Pathological responses to LPS in vivo are diverse. Parenchymal and non-parenchymal cells alike respond to LPS, and a broad array of hemodynamic and metabolic effects of LPS have been implicated in the multiple organ dysfunction that characterizes severe sepsis (16,17). Experimental evidence suggests that one particular type of host response that may be of major importance in LPS-mediated pathology is the LPS-stimulated generation of the inflammatory cytokine cascade. Numerous pro- and anti-inflammatory cytokines are produced following the development of endotoxemia, prominently including tumor necrosis factor (TNF), IL-1, IL-6, IL-8, and IL-10 (18-25). Of these, the direct administration of TNF or IL-1 to animals produces the clinical sepsis syndrome (26-28), thus suggesting that these cytokines may be significant early mediators of LPS activity. In animals, interfering with these cytokines has been shown to confer protection against LPS (29,30), although anti-cytokine therapy has not clearly produced clinical benefit in human studies (31,32).

We have used TNF production as a marker of LPS biological activity in vivo to investigate the mechanism of Hb enhancement of LPS-mediated lethality. To test the hypothesis that Hb infusion may augment the host's pro-inflammatory response to LPS, we compared plasma concentrations of TNF following administration of LPS to mice in the absence and presence of circulating Hb. In addition, we investigated whether pretreatment of mice with Hb resulted in sensitization of TNF producing cells (circulating and fixed tissue macrophages) to subsequent ex vivo stimulation by LPS.

MATERIALS AND METHODS

Animals. Female Swiss Webster mice (28-32 gm) were purchased from Simonsen (Gilroy, CA). Animals were given standard mouse chow and water ad lib throughout the experiments. This study was approved by the Institutional Review Board for the care of animal subjects at the Veterans Administration Medical Center, San Francisco, CA. The investigators adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, revised 1985).

Hemoglobin (Hb). Human Hb, α-α crosslinked (>98% crosslinked between the α99 lysine residues) with bis(3,5-dibromosalicyl) fumarate (33), was provided by the Blood Research Detachment, Walter Reed Army Institute of Research (WRAIR), Washington, DC. Hb solutions were 7.5 g/dL in Ringer's acetate, pH 7.4, contained <5% methemoglobin, 0.7 μg/ml phospholipid, 3.6×10^{-5} moles free iron/mole heme (0.0036% of the total hemoglobin iron), and were sterile and essentially endotoxin-free (<100 pg/ml as assessed by LAL assay).

Lipopolysaccharide (LPS). E. coli lipopolysaccharide 055:B5 was obtained from Difco Laboratories (Detroit, MI). Solutions of LPS in sterile, endotoxin-free 0.9% NaCl (Lyphomed, Deerfield, IL) were vortexed and briefly sonicated (3 min) prior to use.

Tumor necrosis factor (TNF) production in vivo. Mice were injected intraperitoneally with 500 μg LPS (0.5 ml) and infused intravenously by tail vein with 60 mg Hb (2 g/kg) (0.87 ml in most experiments). Hb was infused intravenously either 8-10 hr prior to, coincident with, or 10 hr following injection of LPS. In control animals which received only intraperitoneal LPS, sterile, endotoxin-free 0.9% NaCl was

infused intravenously so that all animals in an experiment received equal volumes of parenteral fluid. Similarly, in control animals which received only intravenous Hb, NaCl was injected intraperitoneally.

Plasma tumor necrosis factor (TNF) determinations. Blood (50-70 μl) for TNF levels was serially obtained from the retroorbital plexus in EDTA-containing capillary tubes at various time points after LPS injection, and plasma was prepared by centrifugation of the capillary tubes in a serofuge (International Equipment Co., Needham Heights, MA). Plasma TNF concentrations were determined in duplicate by ELISA according to the manufacturer's instructions (DuoSet TNFα ELISA kit, Genzyme, Cambridge, MA), using recombinant murine TNFα in plasma as the standard. The sensitivity of the assay was 50 pg/ml plasma. TNF was not detected in normal plasma.

Isolation of Kupffer cells. Mouse nonparenchymal cells were isolated from the liver by modifications of the methods of Friedman and Richman et al. (34,35). Briefly, the inferior vena cava of mice that had been anesthetized with methoxyflurane was infused with 8 ml prewarmed 0.26% pronase (Boehringer Mannheim, Indianapolis, IN) in Iscove's Modified Dulbecco's Medium (IMDM, Life Technologies Inc., Grand Island, NY). The portal vein had been previously transected to allow effective perfusion of the liver. The liver was excised, gently minced, and incubated in 50 ml 0.26% pronase for 15 min at 37°C. 0.25 mg DNAse (Sigma, St. Louis, MO) then was added, followed by 30 min additional incubation at 37°C with continuous stirring. A second aliquot of DNAse (0.25 mg) was added; the suspension then was filtered through 74 μm nylon mesh (Small Parts, Miami, FL), and finally was centrifuged at 600 x g for 7 min at room temperature. The cell pellet was resuspended in 2 ml H-Y medium (Sigma) and spun through a discontinuous 8.2% and 16.5% gradient of a triiodinated derivative of benzoic acid (Accudenz, Accurate Chemical and Scientific Corp., Westbury, NY) at 39,000 x g for 30 min at 20°C. The nonparenchymal cells,

which were present at the interface between the two Accudenz layers, were removed, washed once in IMDM by centrifugation at 600 x g for 7 min at room temperature, and then resuspended in 5 ml of H-Y medium supplemented with 10% fetal bovine serum (Gibco BRL, Gaithersburg, MD) and penicillin/streptomycin (Irvine Scientific, Santa Ana, CA). Cells were counted with a hemocytometer, and viability was assessed by trypan blue exclusion. In all our experiments, cells were more than 95% viable. Kupffer cells in the nonparenchymal cell preparations were quantified by carbon particle (India Ink, Difco Laboratories) phagocytosis. Briefly, 5 ml of 1:10 diluted India Ink suspension was added to 250 ml cell suspension and incubated for 5 hr. Cells that clearly contained multiple carbon particles were counted as Kupffer cells. Kupffer cells ranged from 11-14% of total cells in preparations from both normal (untreated) mice and mice previously infused with Hb (see below).

Isolation of peripheral blood mononuclear cells. Approximately 1 ml mouse blood was collected into EDTA by cardiac puncture from anesthetized mice, and centrifuged through Lympholyte M (Cedarlane Laboratories Limited, Hornby, Ontario, Canada) at 400 x g for 30 min at room temperature. Mononuclear cells were collected from the interface, washed once in IMDM by centrifugation at 600 x g for 7 min, and resuspended in 2 ml H-Y medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. Cells were counted with a hemocytometer. On the basis of immunocytochemical staining with a recombinant fusion protein composed of murine M-CSF (monocyte colony-stimulating factor) and alkaline phosphatase (generously provided by Dr. Larry Rohrschneider, Fred Hutchinson Cancer Research Center, Seattle, WA), 3-7% of mouse peripheral blood mononuclear cells obtained from buffy coats are monocytes (36).

Isolation of peritoneal macrophages. Peritoneal macrophages were collected by lavage of the abdominal cavity with 8 ml cold IMDM medium. The cells were centrifuged from IMDM at 600 x g for 7 min at room temperature, and then

resuspended in 2 ml H-Y medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. Cells were counted with a hemocytometer, and macrophages identified by phagocytosis of India Ink carbon particles, as described above for Kupffer cells. By this analysis, 79-83% of peritoneal lavage cells were macrophages.

Ex vivo TNF production by monocytes/macrophages. Mice were infused intravenously by tail vein with 60 mg Hb (0.87 ml) or with an equal volume of endotoxin-free 0.9% NaCl. Ten hr later, Kupffer cells, peripheral blood mononuclear cells, or peritoneal macrophages were isolated as described above and cultured in 96-well tissue culture plates in 0.25 ml H-Y medium supplemented with 10% fetal bovine serum, penicillin and streptomycin. Wells contained 1.25×10^5 non-parenchymal cells (approximately $1.4\text{-}1.7 \times 10^4$ Kupffer cells), 1.25×10^5 peripheral blood mononuclear cells (approximately $0.4\text{-}0.9 \times 10^4$ monocytes), or 0.5×10^5 peritoneal lavage cells (approximately 0.4×10^5 peritoneal macrophages). Cells were allowed to equilibrate for 2 hr in the plates at 37°C in 10% CO_2, and then 2.5 ml E. coli 055:B5 LPS was added per well (final LPS concentrations 1-1000 ng/ml). Five hr later, 50 ml aliquots of the culture medium were removed and frozen at -70°C. They were subsequently assayed for TNF concentration by ELISA according to the manufacturer's instructions (DuoSet TNFa ELISA kit, Genzyme, Cambridge, MA), using recombinant murine TNFa in culture medium as the standard. The sensitivity of the assay was 50 pg/ml culture medium.

Absorption spectroscopy for detection of Hb in peritoneal fluid. The presence of Hb in peritoneal fluid was determined by measurement of absorbance at 405 nm in a plate reader (BioWhittaker Kinetic-QCL reader, Walkersville, MD). This wavelength, part of the Soret peak of Hb, was shown in preliminary experiments to be capable of detecting 0.5 μg/ml Hb.

Statistical analysis. Differences between plasma TNF levels of LPS only and Hb + LPS mice were evaluated by the Mann-Whitney U test. Differences in ex vivo production of TNF by cells from normal and Hb-treated mice, in response to a range of concentrations of LPS in vitro, were evaluated by repeated measures ANOVA.

RESULTS

Effect of Hb on LPS-mediated TNF production in vivo.

In all the experiments detailed below, plasma TNF concentrations were serially determined after intraperitoneal injection of LPS into mice, in the absence or presence of intravenously administered Hb. Following the intraperitoneal administration of 500 μg LPS, plasma TNF was detectable within 30 min (the earliest time examined) (Fig. 1, open circles). Peak levels of TNF were present between 1-1.5 hr after LPS, followed by a gradual decline in plasma concentration. Low levels of plasma TNF were still detectable in a majority of animals 6 hr after LPS administration (concentrations typically <1 ng/ml) and in an occasional animal at 24 hr (data not shown). The intravenous infusion of Hb 10 hr prior to LPS administration (sequential administration) resulted in significantly higher TNF concentrations (approximately 2-fold) (Fig. 1, closed circles) than in the LPS only control animals at 1, 1.5, 2, and 3 hr after LPS injection ($p<.05$ for LPS + Hb vs. LPS alone). At the time of LPS injection, the plasma cell-free Hb concentration was approximately 1 g/dL (10). The TNF peak concentration in the mice which received both Hb and LPS was observed at 1.5 hr after LPS administration. TNF was undetectable (< 50 pg/ml) in control mice infused with either NaCl or Hb alone in the absence of LPS (data not shown). When Hb was infused intravenously immediately following injection of intraperitoneal LPS (simultaneous administration, generating initial plasma cell-free Hb concentrations of 4-5.5 g/dL (10)), a similarly enhanced production of TNF was observed ($p<.05$ for LPS + Hb vs. LPS alone at 1.5, 2 and 3 hr after LPS injection) (Fig. 2). Plasma TNF concentrations in the animals simultaneously given Hb and LPS were approximately twice those of control LPS only animals, and the peak level was slightly later than in controls (1.5 hr vs. 1 hr). In contrast to the enhancement of circulating TNF levels observed if Hb was infused prior to or simultaneously with LPS, infusion of Hb 10 hr after LPS injection (approximately 8-9 hr after the peak of plasma TNF in response to LPS) did not

alter subsequent TNF concentrations (data not shown). Following this delayed infusion of Hb, TNF levels remained low, indistinguishable from control animals that received intravenous NaCl, rather than Hb, 10 hr after intraperitoneal LPS. Therefore, the late administration of Hb did not elicit a second peak of circulating TNF.

In the mice which received sequential administration of Hb (or NaCl) followed by LPS (TNF levels shown in Fig. 1), 48 hr mortality also was monitored. Mortality in mice which received both Hb and LPS was greater than 90%, whereas mortality in mice which received LPS only was 50%. Since the number of 48 hr survivors in the Hb + LPS group was very low, it was necessary to perform many experiments in order to accumulate a sufficient number of samples from this group. For comparison of TNF levels, similar numbers of samples were assayed from the other three groups (i.e., LPS only survivors, LPS only non-survivors, and Hb + LPS non-survivors). Therefore, the data presented do not reflect the true survival rates in the four groups (Fig. 3). Irrespective of the treatment (LPS only or Hb + LPS), the range of peak plasma TNF concentrations (TNF levels 1.5 hr after LPS injection) was indistinguishable between those animals which died before 48 hr compared to those which survived (Fig. 3). Therefore, peak TNF levels did not predict survival.

Effect of LPS or LPS/Hb complexes on TNF production by Kupffer cells in vitro.

The enhanced TNF response associated with the co-administration of Hb and LPS in vivo, compared to the administration of LPS alone, suggested that TNF producing cells (e.g., hepatic Kupffer cells, peripheral blood mononuclear cells, peritoneal macrophages) might have increased responsiveness to LPS in the presence of Hb. This seemed probable since previous studies in our laboratory had demonstrated that LPS potency was greatly increased in LPS/Hb complexes, compared to LPS alone, in a number of different in vitro biological assays (11-15). To examine this possibility, Kupffer cells were isolated from normal mice and placed in culture, as described in Methods, and then incubated with LPS or LPS/Hb pre-

formed complexes (1,4,16,64,250 or 1024 ng/ml LPS, and 3.5 mg/ml Hb pre-incubated 30 min at 37°C prior to addition to Kupffer cells). Unexpectedly, at each of these LPS concentrations incubation of isolated normal Kupffer cells with a mixture of Hb and LPS generated less TNF (range: 11-90% decreased TNF) than that produced by incubation with LPS alone.

Effect of in vivo Hb infusion upon subsequent ex vivo production of TNF.

To test the possibility that RE cells were primed by Hb in vivo for their subsequent stimulation by LPS, mice were infused intravenously with Hb (or NaCl in control mice), and 10 hr after infusion, Kupffer cells, peripheral blood mononuclear cells, or peritoneal macrophages were isolated. The isolated cells were placed in culture, and stimulation of TNF secretion in response to LPS was determined in vitro. Kupffer cell preparations from Hb-infused mice generated more TNF in response to ex vivo stimulation by LPS than cells obtained from control mice (Fig. 4). As shown, the enhanced (approximately 2-fold) TNF response of cells from Hb-infused mice was observed over a broad range of stimulating LPS concentrations. A trend to greater TNF response to LPS in vitro also was observed with peripheral blood mononuclear cells from mice infused in vivo with Hb compared to control mice, although the differences did not reach statistical significance (p-values ranged from .09 to .29) (Fig. 5). Compared to Kupffer cells, however, peripheral blood mononuclear cells produced less TNF in response to LPS. Neither Kupffer cell nor peripheral blood mononuclear cell cultures produced detectable concentrations of TNF in the absence of added LPS (data not shown).

In contrast to the effect of Hb infusion on Kupffer cells, peritoneal macrophages obtained from either Hb-infused or control mice generated equivalent concentrations of TNF in vitro in response to LPS (Fig. 6). The demonstration that this response of peritoneal macrophages was apparently unaffected by intravenous Hb in vivo suggested that macrophages must directly interact with Hb, possibly by clearing and catabolizing Hb, in order to become primed for subsequent LPS

stimulation, and that peritoneal macrophages had not come in contact with Hb. To determine whether intravenously infused Hb directly interacted with peritoneal macrophages, 1 ml peritoneal lavage samples were analyzed by visible absorption spectroscopy for the presence of Hb. Mice infused intravenously with 60 mg Hb did not have detectable Hb ($\geq$ 0.5 μg/ml) in their peritoneal fluid 4 hr after infusion. At this time, 55% of infused Hb had been cleared from the circulation (10), and therefore the presence of as little as 0.0015% of the cleared Hb (0.5 μg of 33 mg total cleared Hb) would have been detected by this experiment.

DISCUSSION

A variety of preparations of cell-free Hb are undergoing numerous clinical trials for evaluation as potential red blood cell substitutes. Hb infusions are generally well-tolerated in normal humans, with only minor vasoactive and gastrointestinal side effects as the remaining current clinical concerns. However, an extensive literature documenting the ability of iron to act as an adjuvant for bacterial growth has raised concern about the safety of Hb in the setting of infection. Whereas in some pre-clinical models Hb has been shown to be safe when co-administered with gram-negative bacteria (37-40), others (5-8) have obtained the opposite results. We have extensively investigated the influence of Hb on the biological activity of LPS, the toxic component of the gram-negative bacterial cell wall, and results from both our in vitro investigations and animal studies have suggested that Hb transfusions may be dangerous in the setting of endotoxemia. Since bacteremia and/or endotoxemia are likely to be present in many patients who would potentially receive Hb after hemorrhage, shock or trauma, we have performed a large series of experiments in which mice have received both LPS and Hb. These studies have demonstrated that a variety of chemically-modified and native Hb solutions are all capable of enhancing LPS-mediated mortality in mice (10). A number of possible mechanisms for this effect of Hb were investigated and subsequently ruled out: Hb did not increase the levels of endotoxemia that resulted from the intraperitoneal administration of LPS or worsen the hypoglycemia associated with LPS, nor did the combination of Hb and LPS damage the normal gastrointestinal and hepatic barriers such that there was translocation of bacteria into the systemic circulation. However, the clearance of particulate carbon was altered in mice treated with the combination of LPS and Hb in comparison to mice that received LPS only, suggesting that altered RES function might contribute to the deleterious effect of Hb during endotoxemia (10).

Cells of the RES are believed to play a major role in the pathophysiology of sepsis due to their ability to produce pro-inflammatory cytokines. In the experiments presented in this report, we investigated the possibility that monocytes and/or macrophages might generate a more pronounced response to LPS as a result of Hb infusion. We chose to monitor LPS-induced TNF production as a marker of initiation of the inflammatory cascade because of its dramatic induction by LPS and its presumed role in mediating the pathophysiological effects of sepsis. Our principal in vivo finding was that Hb infusion enhanced the production of this cytokine in response to LPS. This Hb effect was observed whether Hb was administered coincident with LPS or several hours prior to LPS. Since Hb did not elicit detectable TNF production in the absence of LPS or when administered approximately 10 hours after LPS, we have concluded that Hb was acting to prime TNF-producing cells so that they became hyperresponsive to LPS. This conclusion is consistent with our previous observation that Hb infusion stimulated RES function as determined by the clearance of particulate carbon (10). Amongst the TNF-producing cells in the body, intravenous Hb would potentially interact with circulating peripheral blood mononuclear cells because of their coexistence in blood, and with fixed tissue macrophages, particularly hepatic Kupffer cells, because of the known contribution of the RES to clearance of crosslinked hemoglobin (41). Therefore, we conducted a series of experiments to determine whether these TNF-producing cells became altered after Hb infusion.

In our studies of LPS-induced TNF production by cells ex vivo, Hb was infused intravenously 10 hr prior to harvesting of monocytes and/or macrophages in order to allow sufficient time for interaction of Hb with these cells. When Kupffer cells were placed in culture and then stimulated with LPS in vitro, cells obtained from Hb-treated mice demonstrated an exaggerated TNF response compared to cells obtained from control animals. Since free Hb was not present in the ex vivo culture system, we interpret these results to indicate that Hb infusion in vivo had modified the intrinsic sensitivity of these cells to LPS. TNF produced by LPS-treated

peripheral blood mononuclear cells was very low, and an effect of Hb could not be statistically demonstrated. The lack of difference in sensitivity of peritoneal macrophages from normal and Hb-treated mice was not unexpected because Hb was not cleared into peritoneal fluid. Therefore, our results suggest that the hyperresponsive Kupffer cells probably had physically interacted with Hb. Our initial in vitro experiments, in which we demonstrated that LPS/Hb mixtures incubated with normal Kupffer cells generated less TNF than incubations with LPS alone, suggested that Hb was not directly increasing the biological potency of LPS in vivo via the production of extracellular LPS/Hb complexes. Rather, since the Hb enhancement effect in vivo was observed even when Hb was infused 10 hr prior to LPS, it seemed more likely that clearance of Hb into the reticuloendothelial cell system (RES) might have increased the sensitivity of phagocytic cells of the RES to subsequent stimulation by LPS. Pertinently, another laboratory recently demonstrated that infusion of liposome encapsulated hemoglobin (LEH) induced hyperresponsiveness to LPS in splenocytes, ex vivo, when these cells were obtained 15 hr after in vivo exposure to LEH, but hyporesponsiveness when these cells were obtained only 2-4 hr after administration of LEH (42).

Several lines of evidence suggest that the ability of Hb to produce cell hypersensitivity to LPS may reside in the heme iron component of the molecule. Iron is well known to catalyze cellular redox changes and oxidant damage (43) and is believed to contribute to tissue injury during shock (44; 45). Hypoferremia, which is characteristic of sepsis, may be protective by limiting iron-catalyzed oxidant cell damage as well as by limiting bacterial proliferation. Alterations in the vasculature may be of particular importance during sepsis, and iron has been shown to sensitize endothelial cells to oxidant challenge (46). Oxidant-mediated damage is more pronounced with methemoglobin than with ferrous hemoglobin, is inhibited by scavenging heme, and results in ferritin production, all suggesting a role for released, free iron (47). Another potential role for heme iron in producing an augmented physiologic response to LPS could result from an alteration in nitric

oxide availability. Hemoproteins, including hemoglobin, demonstrate very high affinity binding of nitric oxide (48). Hb can scavenge nitric oxide from LPS-treated vascular tissue in vitro (49), and in vivo causes vasoconstriction and hypertension (43,48,50). Therefore, it is likely that Hb enhances LPS-related processes by diverse processes possibly involving the vasculature as well as the RES.

Our data indicate that the ability of Hb to enhance lethality during endotoxemia may involve, in part, a perturbation of monocytes/macrophages that results in enhanced sensitivity to LPS. The clearest demonstration of increased LPS responsivity after Hb infusion in vivo was with the preparations of Kupffer cells, which are known to respond prominently to LPS with production of TNF. Although these preparations contained cell types (e.g., endothelial cells and lipocytes) other than Kupffer cells, the trend toward enhanced LPS sensitivity shown by preparations of peripheral blood mononuclear cells from Hb-treated mice strongly implicates the macrophage/monocyte cell lineage in this process.

The precise role of augmented production of TNF in providing a basis for the increased mortality associated with infusion of both LPS and Hb remains unclear. Although administration of Hb prior to or coincident with LPS approximately doubled the serum concentrations of TNF induced by LPS, the range in peak TNF levels was equivalent among survivors and non-survivors. This lack of correlation between TNF levels and clinical outcome has been noted previously (51,52), although an important role for TNF in mediating the physiological effects of endotoxemia has been well established. Additionally, we have observed increased lethality when Hb was administered 10 hr following LPS (10), a model which we currently showed does not demonstrate an effect of Hb on serum TNF concentrations. In this latter model, it is difficult to envision that Hb could enhance mortality via a TNF mechanism unless there is an effect of Hb on locally-acting cytokine (i.e., TNF that does not enter the circulation (25)). In our experimental system, it is possible that enhancement by Hb of LPS lethality is via the modification of a function(s) of monocytes/macrophages other than the production of

TNF, levels of which may be a surrogate indicator for alterations of other more important cytokines. Further studies will be needed to define more precisely the role of TNF in this phenomenon.

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FIGURE LEGENDS

Fig. 1. Plasma Tumor Necrosis Factor (TNF) induced by LPS, in the presence of Hb administered prior to LPS. Mice were injected intravenously with Hb (60 mg/mouse) or NaCl, and 10 hr later were injected intraperitoneally with an LD50 dose of LPS (500 mg). Plasma TNF concentrations following LPS administration were determined by ELISA. Data are presented as mean $\pm$ SE of 35 mice (Hb + LPS) and 20 mice (NaCl + LPS). * $p<.05$ (Mann-Whitney U test).

Fig. 2. Plasma Tumor Necrosis Factor (TNF) induced by LPS, in the presence of Hb administered coincident with LPS. Mice were injected intraperitoneally with an LD50 dose of LPS (500 mg), and then immediately were injected intravenously with Hb (60 mg/mouse) or NaCl. Plasma TNF concentrations following LPS administration were determined by ELISA. Data are presented as mean $\pm$ SE of 16 mice (LPS + Hb) and 15 mice (LPS + NaCl). * $p<.05$ (Mann-Whitney U test).

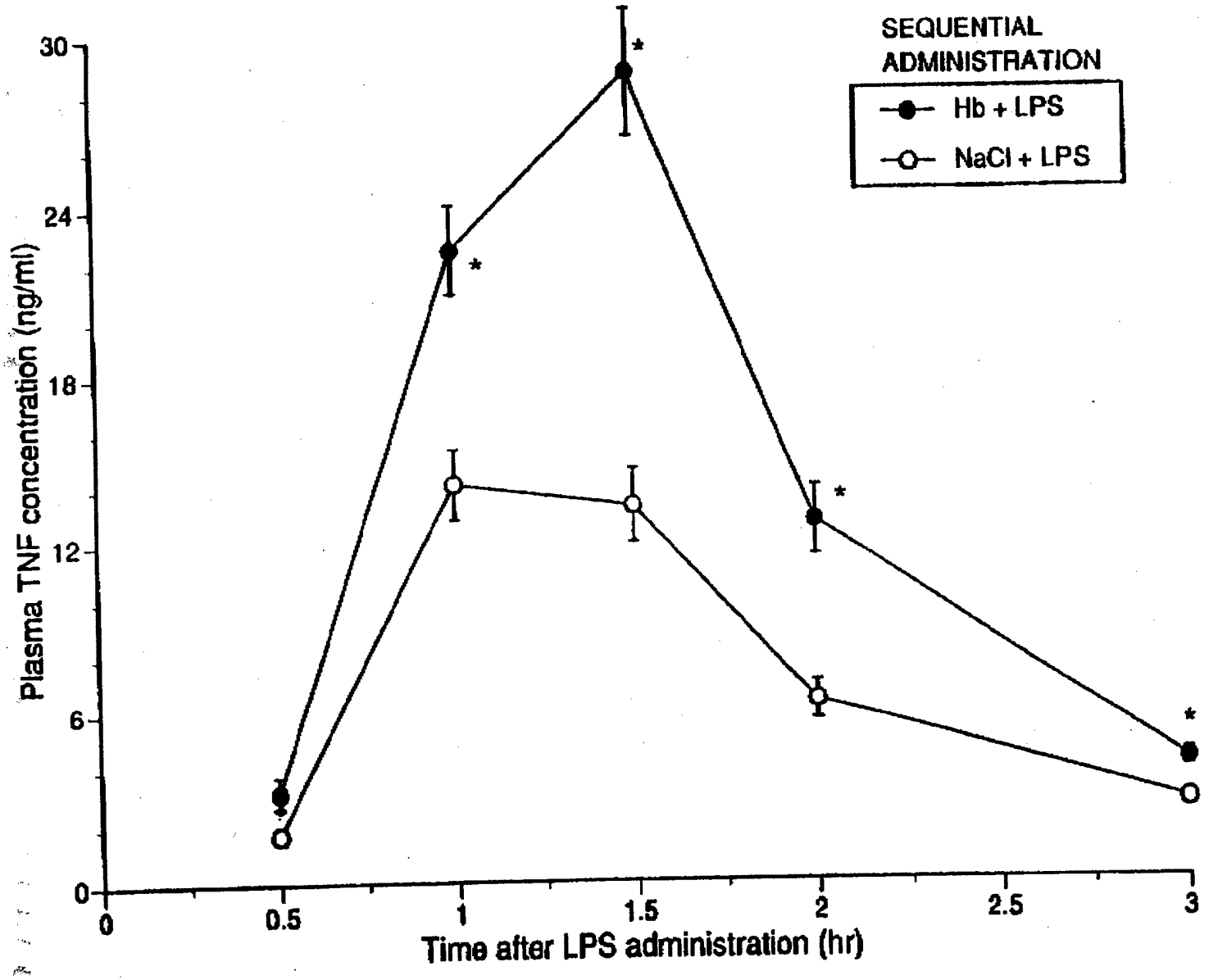
Fig. 3. Peak plasma Tumor Necrosis Factor (TNF) concentrations in surviving vs. non-surviving mice. TNF concentrations at 1.5 hr after injection of LPS are plotted for individual mice which received sequential administration of intravenous Hb (60 mg/mouse) or NaCl, followed 10 hr later by intraperitoneal LPS (500 mg) (see Fig. 1). Mice are grouped according to survival status at 48 hr after LPS. TNF was undetectable (<50 pg/ml) in normal murine plasma.

Fig. 4. Tumor Necrosis Factor (TNF) production by LPS-stimulated Kupffer cells obtained from Hb-infused or control mice. Mice were injected intravenously with Hb (60 mg/mouse) or NaCl. Ten hr later, pronase was injected, the liver was excised and digested with DNase and pronase, and the digested liver preparations were centrifuged on Accudenz to isolate Kupffer cells. The Kupffer cell-enriched preparations were placed in culture and stimulated with LPS; TNF in the culture

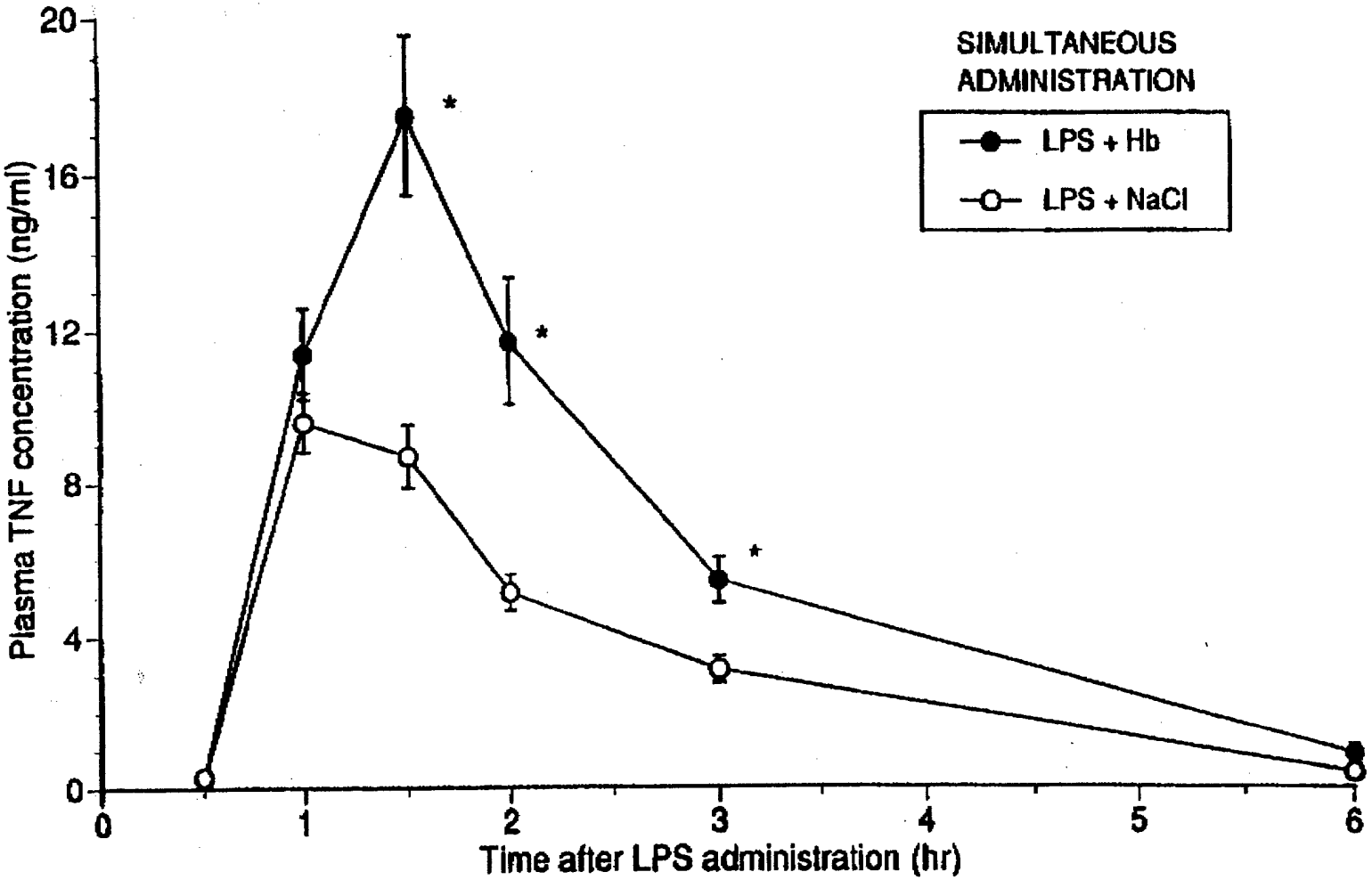
medium was measured by ELISA. Data are presented as mean ± SE of 17-23 measurements at each LPS concentration. Six independent experiments (six Hb-treated and six control mice) were performed and the results pooled. * $p<.05$ (Mann-Whitney U test). The Hb-treated and control groups also were significantly different by repeated measures ANOVA ($p<.05$).

Fig. 5. Tumor Necrosis Factor (TNF) production by LPS-stimulated peripheral blood mononuclear cells (PBMC) obtained from Hb-infused or control mice. Mice were injected intravenously with Hb (60 mg/mouse) or NaCl. Ten hr later, PBMC were isolated, placed in culture, and stimulated with LPS; TNF in the culture medium was measured by ELISA. Monocytes comprised 3-7% of total PBMC. Data are presented as mean ± SE of 8 measurements at each LPS concentration. Four independent experiments (four Hb-treated and four control mice) were performed and the results pooled.

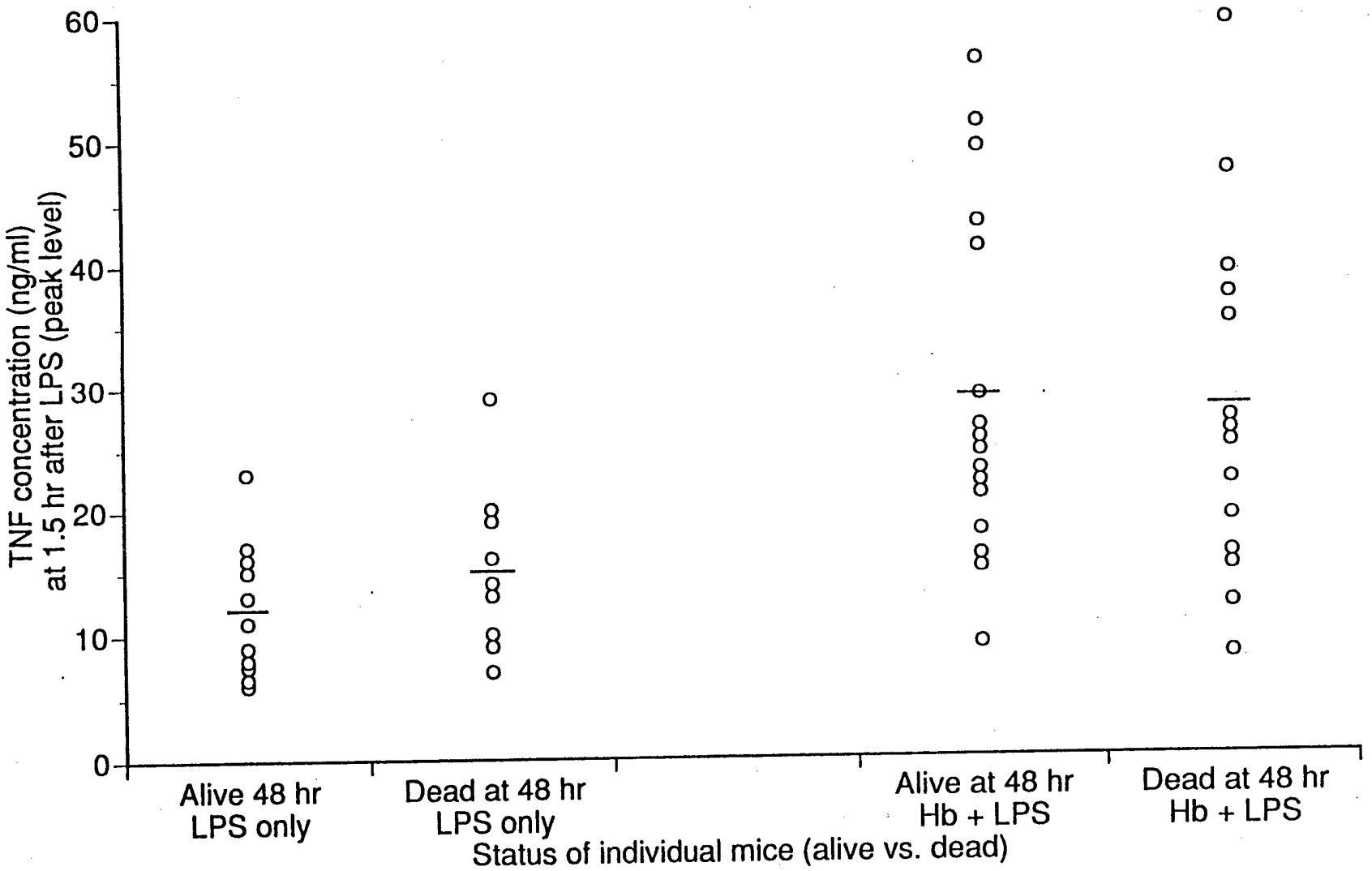
Fig. 6. Tumor Necrosis Factor (TNF) production by LPS-stimulated peritoneal macrophages obtained from Hb-infused or control mice. Mice were injected intravenously with Hb (60 mg/mouse) or NaCl. Ten hr later, peritoneal macrophages were isolated, placed in culture, and stimulated with LPS; TNF in the culture medium was measured by ELISA. Data are presented as mean ± SE of 12 measurements at each LPS concentration in the Hb-treated group and 10 measurements at each LPS concentration in the control group. Six independent experiments (six Hb-treated and six control mice) were performed and the results pooled.

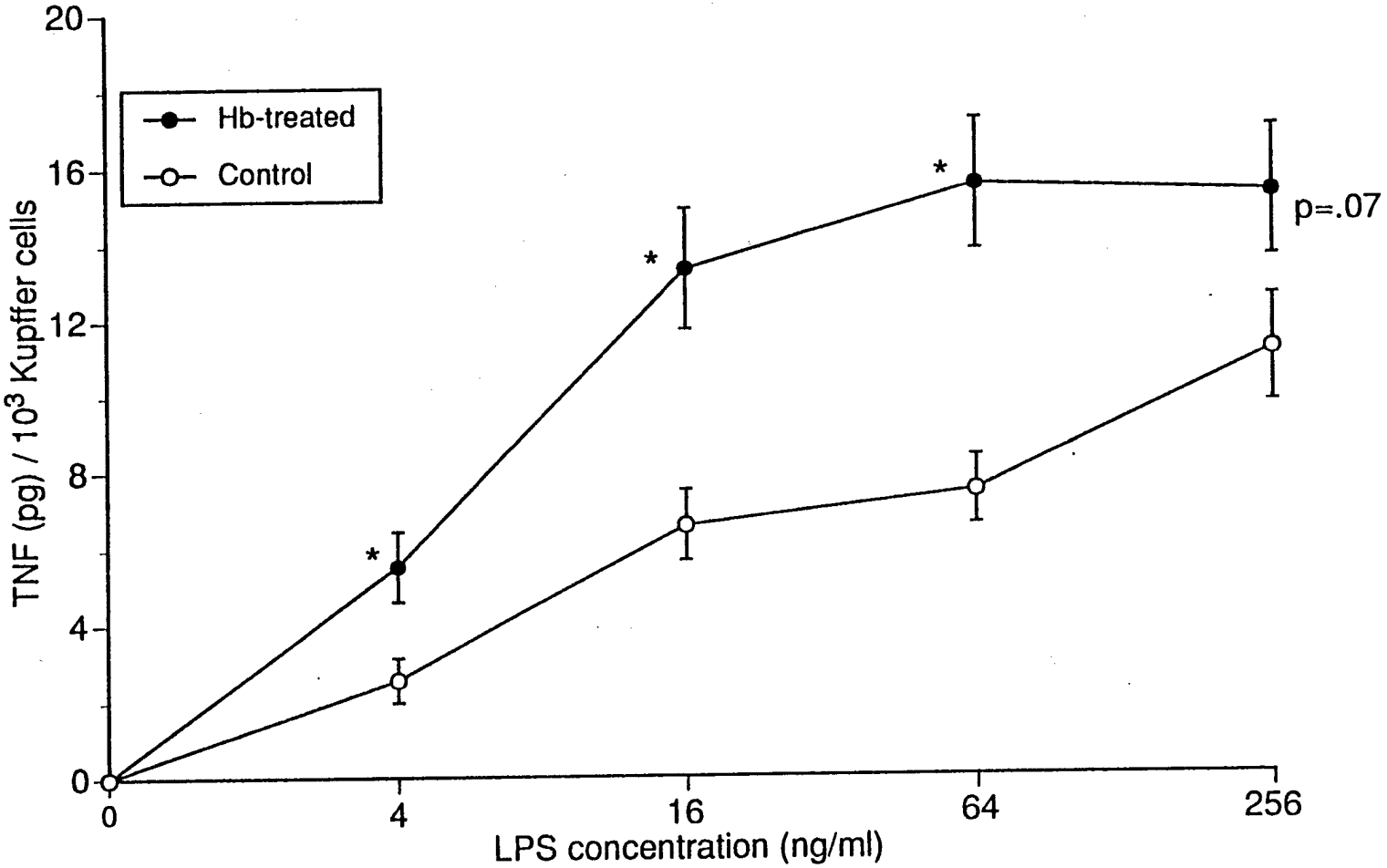

SEQUENTIAL
ADMINISTRATION
Hb + LPS
NaCl + LPS
Plasma TNF concentration (ng/ml)
30
24
18
12
6
0
0
0.5
1
1.5
2
2.5
3
Time after LPS administration (hr)
*
*
*
*

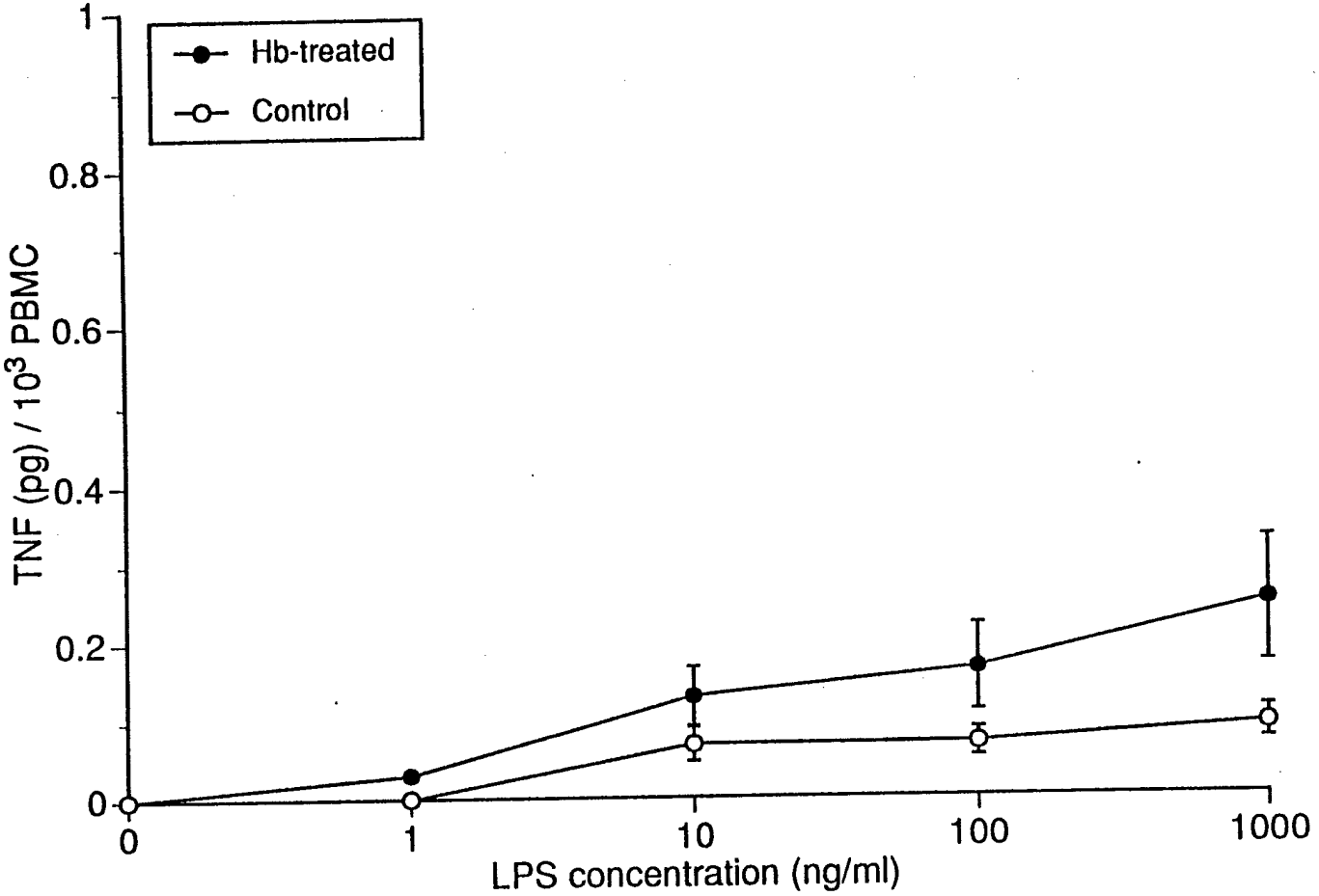
Su et al.
Fig. 1

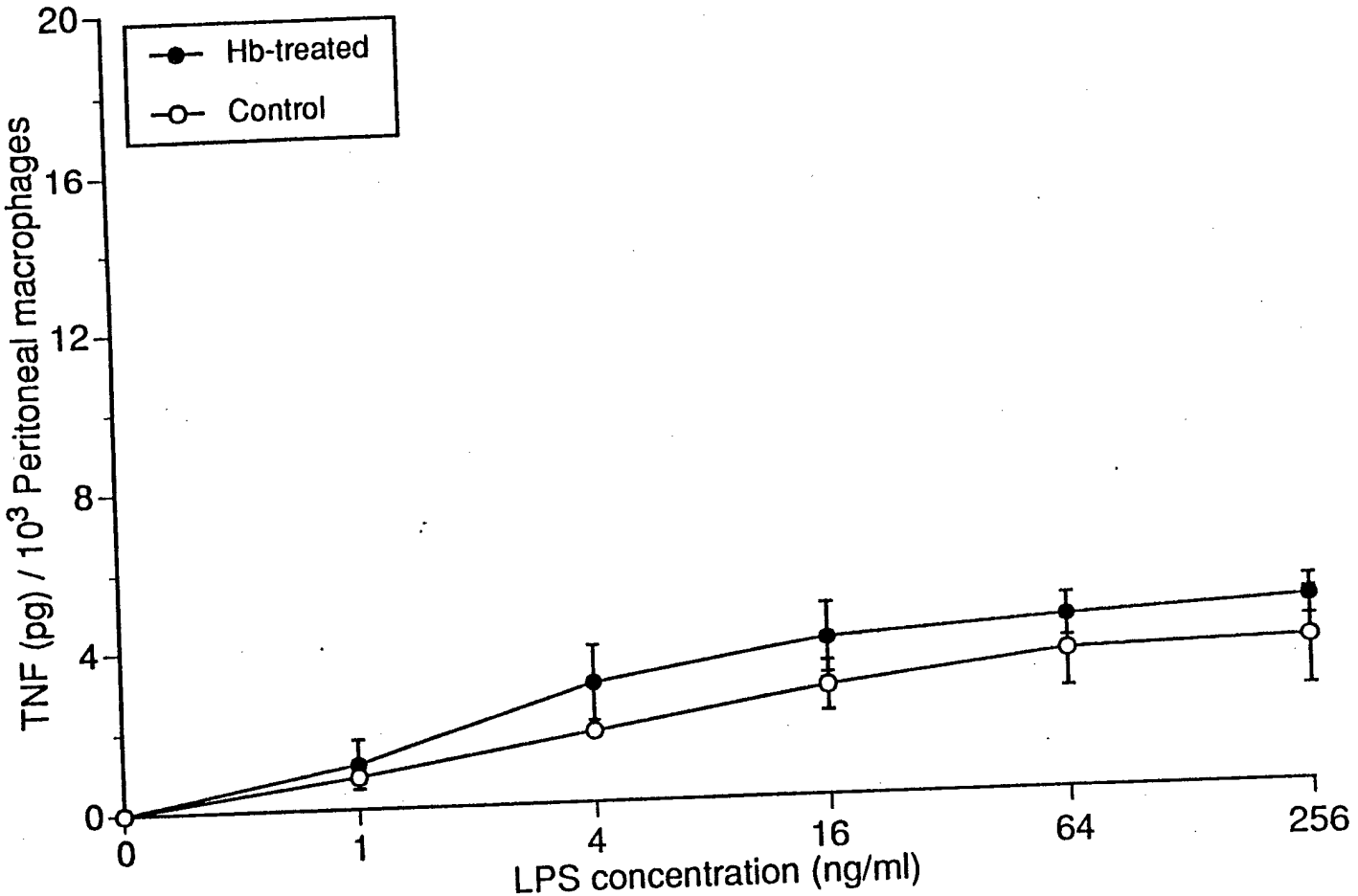

SIMULTANEOUS
ADMINISTRATION
LPS + Hb
LPS + NaCl
Plasma TNF concentration (ng/ml)
20
16
12
8
4
0
0
1
2
3
4
5
6
Time after LPS administration (hr)
*
*
*

Su et al.
Fig. 2


60
50
40
30
20
10
0
TNF concentration (ng/ml)
at 1.5 hr after LPS (peak level)
Alive 48 hr
LPS only
Dead at 48 hr
LPS only
Alive at 48 hr
Hb + LPS
Dead at 48 hr
Hb + LPS
Status of individual mice (alive vs. dead)


20
16
12
8
4
0
TNF (pg) / 10³ Kupffer cells
Hb-treated
Control
*
*
*
p=.07
0
4
16
64
256
LPS concentration (ng/ml)


Hb-treated
Control
TNF (pg) / 10^3 PBMC
1
0.8
0.6
0.4
0.2
0
0
1
10
100
1000
LPS concentration (ng/ml)


Hb-treated
Control
TNF (pg) / 10³ Peritoneal macrophages
20
16
12
8
4
0
0
1
4
16
64
256
LPS concentration (ng/ml)

Bacterial Endotoxins: Basic Science
to Anti-Sepsis Strategies, pages 161–172

HEMOGLOBIN: A NEWLY RECOGNIZED BINDING PROTEIN FOR BACTERIAL ENDOTOXINS (LPS)

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Abbreviations: Hemoglobin (Hb), crosslinked cell-free hemoglobin (ααHb), lipopolysaccharide (LPS), Limulus amebocyte lysate (LAL), tissue factor (TF), mononuclear cell (MNC), human umbilical vein endothelial cells (HUVEC)

ABSTRACT

Administration of purified hemoglobin (Hb) as a cell-free resuscitation fluid is associated with multiple organ toxicities. Many of these toxicities are characteristic of the pathophysiological effects of bacterial endotoxins (lipopolysaccharide, LPS). To better understand the potential role of LPS in the observed in vivo toxicities of Hb, we examined mixtures of Hb and LPS for evidence of LPS-Hb complex formation. LPS-Hb complexes were demonstrated by three techniques: ultrafiltration through 300 kDa cut-off membranes, which distinguished LPS in complexes (87-89% <300 kDa) from LPS alone (90% >300 kDa); density centrifugation through sucrose, which distinguished denser LPS alone from LPS-Hb complexes; and precipitation by 67% ethanol, which demonstrated 2-3 fold increased precipitability of Hb in complexes compared to Hb alone. Interaction of LPS with Hb was also associated with markedly increased biological activity of LPS, as manifested by enhancement of LPS activation of Limulus amebocyte lysate (LAL), increased release of human mononuclear cell tissue factor, and enhanced production of human endothelial cell tissue factor. These results demonstrated that hemoglobin can serve as an endotoxin binding protein, and that this interaction results in the alteration of several of the physical characteristics of LPS and enhancement of the biological activities of LPS. These findings suggest that a mechanism for the toxicity of infused Hb in vivo may involve potentiation of the biological effects of LPS. In addition, these observations suggest a mechanism by which LPS-related morbidity during sepsis could be enhanced by erythrocyte hemolysis.

INTRODUCTION

Several studies have been conducted using cell-free preparations of purified human hemoglobin (Hb) that were developed for use as a cell-free resuscitation fluid (DeVenuto, Zegna, 1982; Sehgal et al., 1984; Winslow, 1989). Preliminary

experiments in our laboratory have suggested that human hemoglobin may have a physiologically important interaction with endotoxin (lipopolysaccharide, LPS). Previously, an association between LPS and Hb had been suggested by the observations that in vivo administration of Hb produced the following toxicities: activation of the complement and coagulation cascades (Feola, et al., 1988a; Feola, et al., 1988b; Marks, et al., 1989), disseminated intravascular coagulation with resultant thrombosis (Feola, et al., 1988a; White, et al., 1986a; White, et al., 1986b), ischemic parenchymal damage (Feola, et al., 1988a; Marks, et al., 1989), hypertension and bradycardia (Amberson, et al., 1949; Savitsky, et al., 1978), a decrease in glomerular filtration rate and renal plasma flow (Brandt, et al., 1951), and mild prolongations of the partial thromboplastin time (Savitsky, et al., 1978). Since these toxicities can in large part be explained by the known consequences of endotoxemia, and since the presence of LPS in preparations of Hb utilized for in vivo studies has been documented (Feola, et al., 1988a; Marks, et al., 1989), it has been proposed that LPS has a contributory role in the observed in vivo toxicity of Hb infusions (White, et al., 1986a; White, et al., 1986b). Previously, Hb and LPS have been shown to produce synergistic in vivo toxicity (White, et al., 1986b), and we have demonstrated that Hb is capable of enhancing the procoagulant activity of LPS in vitro (Roth, Levin, 1994). Therefore, we hypothesized that Hb binds LPS, and that the interaction between these molecules could alter the biological activity of LPS. The present study was designed to determine whether complex formation occurs between Hb and LPS, and evaluate the ability of Hb to alter biologic activities of LPS.

MATERIALS AND METHODS

Reagents. Sterile, 15 ml Falcon tubes were obtained from Becton Dickinson (Mountainview, CA). Sterile, endotoxin-free water and 0.9% NaCl were purchased from Travenol Laboratories (Deerfield, IL).

Glassware. Glassware was heated at 190°C in a dry oven for 4 hours.

Hemoglobin. Human Hb, covalently crosslinked between α chains (ααHb) with bis(3,5-dibromo-salicyl) fumarate as described previously (Winslow, et al., 1991; Chatterjee, et al., 1986) in order to stabilize the protein's tetrameric structure, and purified non-crosslinked human A_0 (HbA_0) prepared by ion exchange HPLC of purified human Hb as described previously (Christensen, et al., 1988), were provided by collaborators at the Blood Research Division of the Letterman Army Institute of Research (BRD/LAIR), San Francisco, CA. Hb preparations contained less than 0.4 EU/ml endotoxin (referenced to E. coli lipopolysaccharide B, O55:B5, Difco Laboratories, Detroit, MI), as determined by the Limulus amebocyte lysate (LAL) test (Levin, Bang, 1968).

Endotoxin. E. coli O26:B6 lipopolysaccharide (LPS) was obtained from Difco Laboratories (Detroit, MI). ^{14}C-LPS (Salmonella typhimurium PR122(Rc)) was purchased from List Biologicals, Inc. (Campbell, CA) and was resuspended in endotoxin-free water at 1 μCi/ml (1 mg/ml). Proteus mirabilis LPSs (smooth strains S1959 and 03; rough mutant R110; and deep rough mutant R45 LPS) were provided by collaborators at the Institute of Microbiology and Immunology, University of Lodz, Poland.

Limulus amebocyte lysate (LAL). Amebocyte lysates were prepared from Limulus polyphemus (the North American horseshoe crab) by disruption of washed amebocytes in distilled water (Levin, Bang, 1964; Levin, Bang, 1968).

Chromogenic substrate. Chromogenic substrate S-2423 (AB Kabi Vitrum, Molndal, Sweden) was the gift of Dr. Petter Friberger.

Chromogenic Limulus amebocyte lysate (LAL) test. 50 µl of sample and 30 µl of LAL (freshly diluted 1:20 in 0.9% NaCl prior to use) were incubated in tissue culture plates for 30 min at 37°C in a temperature-controlled plate reader (Kinetic-QLC, Whittaker Bioproducts Inc., Walkersville, MD). 40 µl chromogenic substrate S-2423 (0.25 mM, in 25 mM Tris, pH 8.6) was added to each well, mixtures were incubated at 37°C for 5 min, and absorbances at 405 nm then were determined.

Ultrafiltration. Solutions of Hb were prefiltered through an endotoxin-free 300 kDa membrane prior to use to remove aggregated protein particles. Hb (100 µg/ml) was incubated with E. coli O26:B6 LPS (50 µg/ml) for 30 min at 37°C. Mixtures then were filtered manually with a 3 ml syringe (according to the directions of the filter manufacturer) at room temperature, using a 300 kDa cut-off filter (ultrafree-PFL polysulfone 300, Millipore Corporation, Bedford, MA). LPS concentrations in filtered solutions of Hb, Hb and LPS mixtures, or LPS alone were determined by the chromogenic LAL test (described above), using starting mixtures of Hb-LPS, or LPS alone, for the standard curve. Hb protein concentrations were determined by the BCA protein assay (Pierce, Rockford, IL).

Sucrose centrifugation of LPS and Hb. ^{14}C-S. typhimurium LPS (0.005 µCi) was added to each of the Hb solutions (each diluted to 10 mg/ml), and the mixtures were incubated for 30 min at 20°C. Aliquots of LPS-Hb mixtures, LPS alone, or Hb alone then were layered over 5% pyrogen-free sucrose and centrifuged at 2,900 x g for 30 min at 20°C, in a Sorvall RC-5 centrifuge (Du Pont Instruments, Wilmington, DE). Scintillation counting was performed, after samples were diluted 10-fold in fluor (Formula A-989, NEN Research Products, Boston, MA), in a Tracor Analytic Liquid Scintillation System (Tracor Analytic, Elk Grove Village, IL). For samples containing Hb, quenching of ^{14}C-LPS by Hb was reversed as follows: 0.1 ml aliquots of fractions were diluted ten-fold in water (to 1 ml final volume), and 1 ml Solvable (NEN Research Products, Boston, MA) was added. These mixtures were incubated at 60°C for one hr, and then 0.3 ml 25% H_2O_2 was added.

In other experiments, aliquots of LPS-Hb mixtures, LPS alone, or Hb alone were layered over a sucrose gradient (4-20%) and centrifuged at 52,000 x g for 4 hr at 20°C, in a Sorvall RC70 centrifuge and T641 swinging bucket rotor (Du Pont Instruments, Wilmington, DE). After centrifugation, fractions through the gradient were obtained and analyzed for ^{14}C-LPS and Hb, as described above.

Ethanol precipitation of Hb and LPS-Hb mixtures. Hb (2 µg) was incubated with E. coli O26:B6 LPS (25 µg) in microtiter plate wells for 30 min at 4°C, 20°C or 37°C. Ethanol then was added to each well (final concentration, 67%), and after an additional 30 min the

mixtures were centrifuged at 800 x g for 30 min. The concentrations of Hb in the sediments were determined by protein assay, and LPS concentrations by the phenol-concentrated H_2SO_4 method (Nowotny, 1979).

Mononuclear cell (MNC) tissue factor (TF) assay. E. coli LPS (100 ng/ml) was incubated with Hb (range 0.6-60 mg/ml) for 30 min at 37°C. LPS alone or LPS-Hb mixtures were then incubated for 20 hr at 37°C with human peripheral blood MNC (Rickles, et al., 1977) and assayed for TF with a one-stage coagulation assay (Rickles, et al., 1979). A clotting time of 30 sec was defined as equal to 100 units TF activity (Korn, et al., 1982).

Endothelial cell tissue factor (TF) assay. Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics (San Diego, CA) and cultured in media (containing 2% serum) obtained from Clonetics. Cells were grown to confluent monolayers in sterile 96-well tissue culture plate wells (Nunclon, Applied Scientific, San Francisco, CA). E. coli LPS alone or LPS-Hb mixtures were added to the media in each well (final concentrations: 100 ng/ml LPS; 0.1-10 mg/ml Hb), and incubated for 4 hr. Wells were then washed with media (x 3) and the HUVEC were freeze-thawed (x 2) and sonicated in phosphate buffered saline. To each well then was added human citrated plasma and calcium (25 mM), and plates were incubated for 8 min in a temperature-controlled (37°C) plate reader (Kinetic-QLC, Whittaker Bioproducts Inc., Walkersville, MD). Turbidity was measured at 340 nm, and TF activity was calculated from a standard curve established with rabbit brain thromboplastin (Baxter Corporation, Miami, FL). The turbidity generated at 8 min by 1:100 diluted thromboplastin was arbitrarily defined as 10 TF units.

RESULTS

Demonstration of LPS-Hb complexes. Ultrafiltration experiments demonstrated that 97% of the LPS in LPS-$\alpha\alpha$Hb mixtures and 94% of the LPS in LPS-HbA_0 mixtures were filterable through the 300 kDa membrane, whereas only 16% of LPS alone was filterable (Fig. 1). Approximately 90% of the total Hb protein in each of these LPS-Hb mixtures, and from filtrates of Hb alone, was detected in filtrates (data not shown). Using 100 kDa cutoff membranes, 64% and 72% of LPS in $\alpha\alpha$Hb or HbA_0 mixtures, respectively, were filterable (data not shown). These results indicated that Hb caused the dissociation of LPS into lower molecular weight particles. Utilizing ethanol precipitation, greater than twice the amount of each Hb was precipitated at 20°C in the presence of LPS than was with Hb alone (Fig. 2). In both the absence and presence of Hb, approximately 90% of LPS was precipitated by ethanol (data not shown). Similar 2-3 fold increases in precipitated Hb in the presence of LPS were demonstrated at 4°C and 37°C (data not shown). These results suggested that Hb and LPS formed stable complexes. Following centrifugation in 5% sucrose, 93% of LPS sedimented into the sucrose cushion (bottom fraction) in the absence of protein, whereas only 9% sedimented in the presence of HbA_0 and 13% in the presence of $\alpha\alpha$Hb (Figure 3). Conversely, only 7% of LPS alone remained above the sucrose layer, whereas in the presence of Hb, 87-91% of LPS remained in the top layer. No detectable Hb entered the sucrose layer in either the absence or presence of LPS. Therefore, Hb decreased the density of LPS, resulting in the co-migration of Hb and LPS. When LPS-$\alpha\alpha$Hb mixtures were centrifuged through a 4-20% sucrose gradient, the two

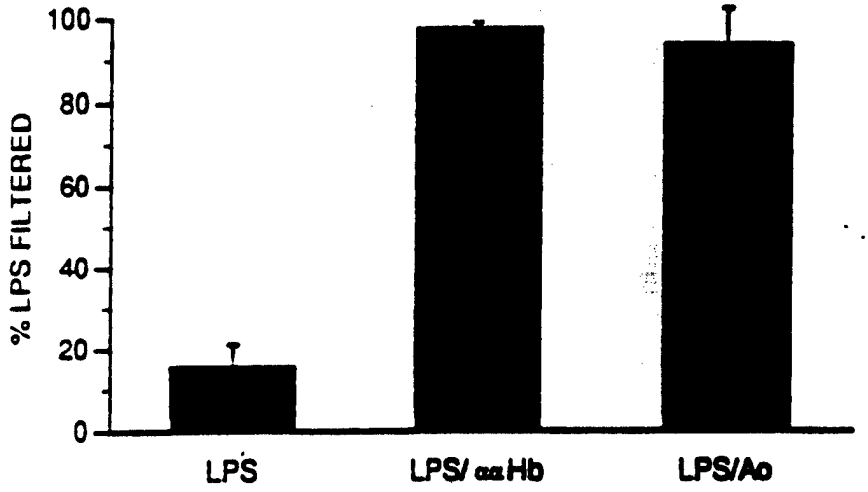

Fig. 1. Ultrafiltration of Hb and LPS. E. coli LPS was incubated with ααHb or native HbA_0, and the mixtures were then filtered through a 300 kDa cut-off ultrafiltration membrane. The percent of LPS filtered, in the absence and presence of Hb, was determined by the LAL test. Presented are the means and 1 S.D. of 3 experiments. Both of the Hb preparations greatly increased the filterability of LPS.

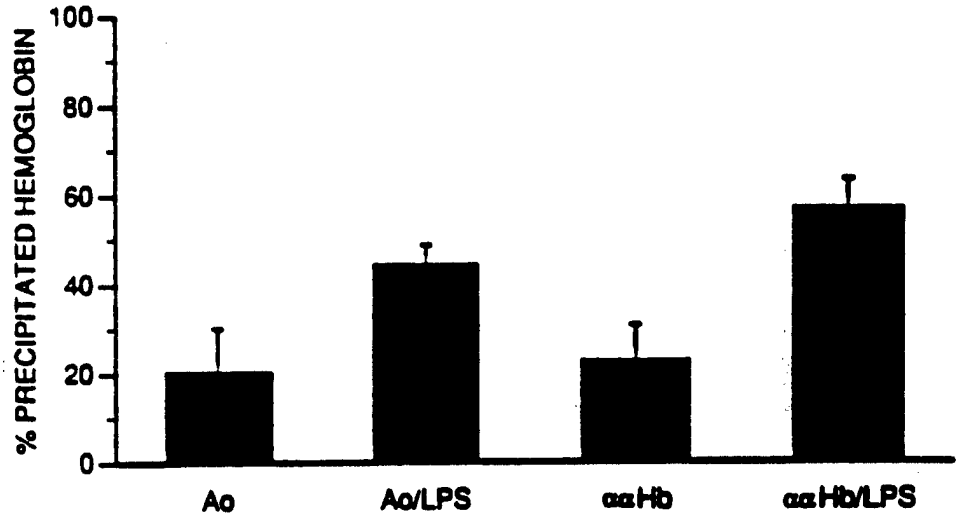

Fig. 2. Precipitation of Hb by ethanol. ααHb or native HbA_0 was incubated with E. coli LPS, and the LPS-Hb complexes or Hb alone were then precipitated from the mixtures by 67% ethanol and sedimented by centrifugation. The quantities of Hb in the sedimented material were determined by protein assays. Presented are the means and 1 S.D. of 8 experiments. Both of the Hb preparations demonstrated increased precipitability in presence of LPS.

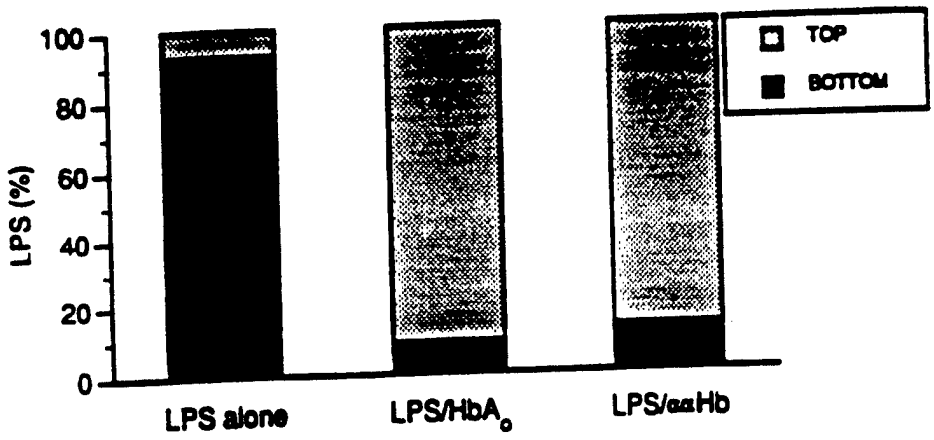

Fig. 3. Centrifugation of Hb and LPS through sucrose. ^{14}C-S. typhimurium LPS was incubated with αα Hb or native HbA$_0$. These LPS-Hb complexes, or LPS in NaCl, were then centrifuged through 5% sucrose. The distribution of radiolabeled LPS was determined in top (stippled columns) and bottom (solid columns) zones of the centrifuged samples. Presented are the means of 4 experiments. LPS alone distributed predominantly in the bottom zone. LPS in the presence of Hb distributed predominantly into the top zone, indicating a decrease in LPS density. Hb remained in the top zone in the presence or absence of LPS. Both of the Hb preparations co-migrated with LPS.

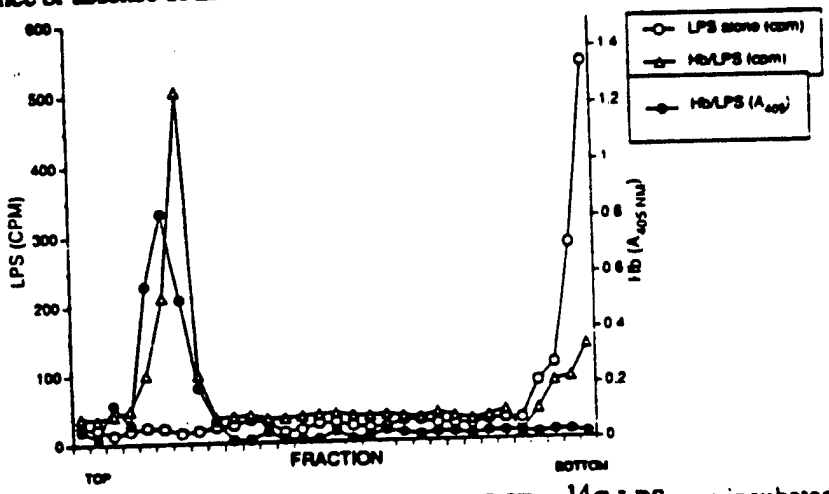

Figure 4. Sucrose density centrifugation of LPS-Hb. ^{14}C-LPS was incubated with αα Hb, and the mixture or LPS alone was centrifuged through a 4-20% continuous sucrose gradient. 0.4 ml fractions were assayed for hemoglobin by absorbance at 405 nm (●), and for LPS by scintillation counting (○, LPS alone; △, LPS in LPS-Hb complexes). The density of LPS was decreased in the presence of Hb, and Hb and LPS co-migrated by density.

components co-migrated part way through the gradient, whereas LPS alone sedimented to the bottom of the tube (Fig. 4). Hb alone sedimented at a rate similar to the Hb-LPS

complexes (data not shown). These results demonstrated that LPS density was decreased in the presence of Hb, and suggested that LPS was disaggregated by Hb.

Biological activity of LPS in Hb-LPS complexes. Hb increased the biological activity of LPS in three independent assays. Firstly, LPS in the presence of $\alpha\alpha$Hb produced enhanced activation of LAL (33-180 fold) compared to LAL activation by LPS alone (Fig. 5). The enhanced LPS biological activity was most prominent with the smooth

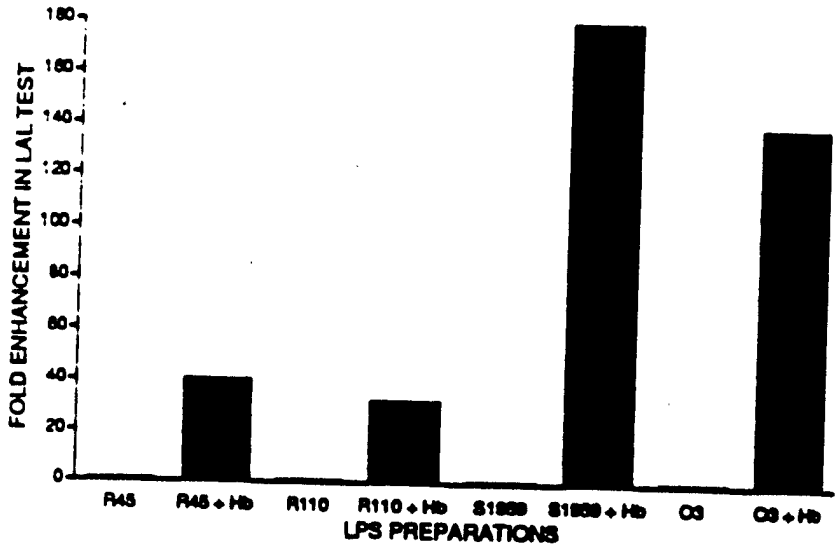

Fig. 5. Enhancement of LPS activation of Limulus amebocyte lysate by Hb. LPSs from P. mirabilis isolates (deep rough R45, rough R110, and smooth S1959 and O3) were incubated with LAL, in the presence and absence of $\alpha\alpha$Hb, and relative LAL reactivities were determined. The reactivity of each LPS alone has been normalized to 1 in order to compare Hb enhancement effects. Presented are the means of 8 experiments. Hb dramatically enhanced the biological activity of each LPS.

Proteus LPSs S1959 and 03, although substantial enhancement was also demonstrated with the rough (R110) and deep rough (R45) mutants. Enhancement of the biological activities of these LPSs also was observed with HbA_O, and the enhancement effect of each Hb was concentration dependent (data not shown). Secondly, LPS-$\alpha\alpha$Hb complexes resulted in greater TF production by human MNC than from MNC following incubation with LPS alone (Fig. 6). The enhancement in TF production was Hb concentration-dependent, ranging from 2-fold at 0.6 mg/ml Hb to 22-fold at 60 mg/ml Hb. Thirdly, $\alpha\alpha$Hb produced an increase in HUVEC TF activity compared to the TF generated by LPS alone, as demonstrated by the increased rate of production of turbidity (absorbance at 340 nm)(Fig. 7). The enhancement effect was Hb concentration-dependent, and LPS-induced

TF activity, as quantified using brain thromboplastin in the standard curve, increased 8-fold in the presence of 10 mg/ml αα-Hb. The enhancement effect was totally inhibited by

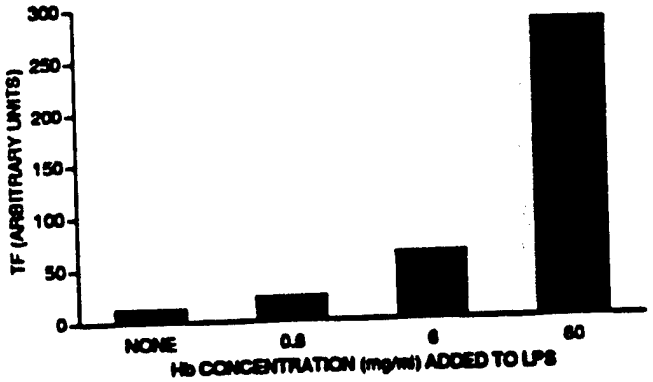

Fig. 6. Effect of Hb on the LPS-induced stimulation of tissue factor procoagulant activity from human peripheral blood mononuclear cells. E. coli LPS (100 ng/ml), in the absence or presence of various concentrations of αα-Hb, was incubated with human mononuclear cells, and tissue factor activity was measured with a plasma clotting assay. Hb enhanced, in a concentration-dependent manner, LPS-stimulated TF production.

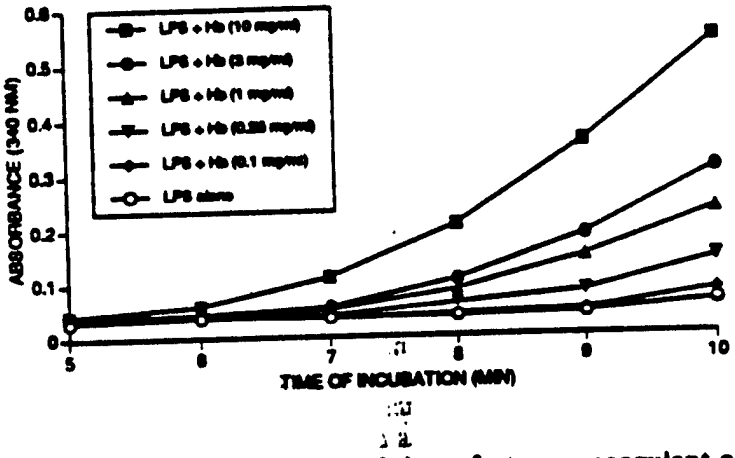

Fig. 7. Effect of LPS on the production of tissue factor procoagulant activity by cultured human endothelial cells. Monolayers of cultured human umbilical vein endothelial cells were incubated with E. coli LPS (100 ng/ml) for 4 hrs in the absence or presence of various concentrations of αα-Hb. The cells were then washed and freezed/thawed twice. Normal human citrated plasma and calcium were added, and clot formation was measured as increased turbidity (A340nm). Tissue factor procoagulant activity was detected as an increased rate of increase in turbidity compared to the clotting rate of recalcified plasma alone. Hb caused a concentration-dependent increase in LPS-stimulated endothelial cell tissue factor activity.

protein synthesis inhibitors cycloheximide and actinomycin D (data not shown). Similar Hb concentration-dependent enhancement of LPS-induced TF in HUVEC was observed with HbA_0 (data not shown). LPS-induced TF protein, as measured with a sensitive ELISA assay, similarly was enhanced by Hb in a concentrtion-dependent manner.

DISCUSSION

In order to investigate the potential role of LPS contamination in the observed toxicities of infused Hb, we performed experiments to determine whether Hb interacted with LPS. Ultrafiltration demonstrated that the molecular weight of LPS (typically $>10^6$ in aqueous solution) was reduced to < 300 kDa in the presence of Hb, and that LPS and Hb co-filtered. The majority of LPS (64-72%) also was <100 kDa in the presence of Hb. Utilizing centrifugation through sucrose, we showed that the density of LPS in the presence of Hb was distinctly less than that of LPS alone, and that LPS and Hb co-migrated. The decrease in LPS density is further evidence for LPS disaggregation. Measurement of Hb precipitation by ethanol indicated that LPS greatly increased the precipitability of Hb, a result which further provided evidence of complex formation. Therefore, our experiments demonstrated that the physical characteristics of both Hb and LPS were altered in LPS-Hb mixtures. These results are consistent with the formation of stable complexes, and establish the ability of hemoglobin to act as an endotoxin-binding protein. Because these results were observed with both crosslinked Hb ($\alpha\alpha$Hb) and unmodified hemoglobin (A_0), we have demonstrated that LPS-binding is an intrinsic property of hemoglobin.

The formation of LPS-Hb complexes was associated with major changes in the procoagulant activities of LPS. Hb enhanced the ability of LPS to initiate coagulation as demonstrated with three independent assays: 1) direct activation of a proteolytic coagulation cascade, as shown with Limulus amebocyte lysate, 2) stimulation of TF production from human MNC, and 3) stimulation of TF production from HUVEC. Enhancement by Hb of LPS procoagulant activity by one or all of these mechanisms may contribute to the toxicities associated with Hb infusions in resuscitation experiments. This enhanced procoagulant activity of LPS may be the etiology of the observed thrombosis and ischemic damage associated with Hb infusion in animals (Feola, et al., 1988a; Marks, et al., 1989), and may also provide a mechanism for some aspects of the synergistic toxicity between Hb and LPS reported previously (White, et al., 1986b; Litwin, et al., 1963). Interestingly, other proteins that are known to bind LPS with a resultant change in LPS biological activity, e.g., melittin (David, et al., 1992), lysozyme (Ohno, Morrison, 1989), complement (Galanos, et al., 1971) or the polypeptide polymyxin B (Morrison, Jacobs, 1976), cause a decrease in LPS toxicity.

In contrast to the increased biological activity we observed for LPS that had been disaggregated and bound to Hb, the process of LPS disaggregation in plasma resulting primarily from its interaction with high density lipoproteins (Ulevitch, et al., 1979) results in detoxification. It is possible that the process of LPS-Hb complex formation might compete in vivo with the process of the LPS-lipoprotein interaction, and therefore potentially interfere with LPS detoxification in plasma. The combination of decreased detoxification of LPS in plasma and enhancement of LPS biological activity secondary to binding to Hb and disaggregation of LPS micelles would magnify the consequences of endotoxemia. In addition to accounting for some of the toxicity observed with Hb infusions, the Hb-LPS interaction also may provide a mechanism by which LPS-induced

intravascular hemolysis during sepsis potentiates the pathophysiologic consequences of endotoxemia.

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